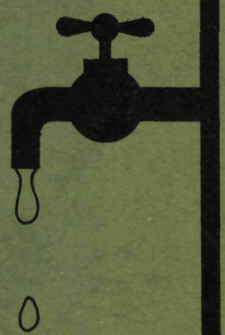
TRACE ELEMENT ABSORPTION AND RETENTION STUDIES IN MICE

The role of experimental conditions and the influence of Ca and Mg



A. A. van Barneveld

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The role of experimental conditions and the influence of Ca and Mg

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The studies presented in this thesis were performed in the Section Nuclear Biotechnique (head Dr. C.J.A. Van den Hamer), Department of Radiochemistry (head Prof. ir. J.P.W. Houtman, at present Prof. dr. ir. J.J.M. de Goey), Interuniversity Reactor Institute, Delft, The Netherlands.

The studies were supported by grant R712 from the Dutch Ministry of Public Health and Environmental Hygiene, at present Ministry of Welfare, Health and Cultural Affairs.

TRACE ELEMENT ABSORPTION AND RETENTION STUDIES IN MICE
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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE GENEESKUNDE AAN DE
KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J.H.G.I. GIESBERS,
VOLGENS HET BESLUIT VAN HET
COLLEGE VAN DEKANEN IN HET
OPENBAAR TE VERDEDIGEN OP
VRIJDAG 13 JANUARI 1984
DES NAMIDDAGS TE 2 UUR PRECIES

DOOR

ABRAHAM ALBERTUS VAN BARNEVELD
GEBOREN TE LEIDEN

1983
Fotodruck J. Mainz, Aachen

"Many do not know that we are here in this world to live in harmony. Those who know this do not fight against each other."

(Dhammapada, old Bhuddist scripture, 3rd cent. B.C., Engl. transl. J. Mascaró)

Aan allen die ik tijdens het schrijven van dit proefschrift tekort gedaan heb

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A.A. Van Barneveld & C.J.A. Van den Hamer

Accepted for publication in:
Nutr. Rep Int. (1984), 29 (January issue).

PAPER VII:
Influence of Ca and Mg on metabolism of Zn, Cu, Co, Cd and Se in mice.
A.A. Van Barneveld, C J A. Van den Hamer & J.P W Houtman

Published in.
Tr. Subst. Environm. Hlth (1982), 16, 196-204.
(Title: Drinking water hardness, trace elements and cardiovascular diseases: Influence of Ca and Mg on metabolism of Cd, Co, Cu, Zn and Se in mice)

APPENDIX I:
De invloed van fytaat in de voeding: een overzicht.
A.A. Van Barneveld

Published in.
Voeding (1982) 44, 51-58.

APPENDIX III.
Main effects of Ca and Mg on metabolism of Mn, Cd and Pb in mice.

Accepted for publication in:
Proc Int Symp "Health effects and interactions of essential and toxic elements", June 1983, Lund, Sweden.

PREFACE

In 1972 the "Ministerie van Volksgezondheid en Milieuhygiene" (Dutch Ministry of Public Health and Environmental Hygiene) asked the "Gezondheidsraad" (Health Council of The Netherlands) to assess the importance of possible adverse effects of central softening of drinking water on public health. The interest in this subject was due to the mentioning of a negative correlation between drinking water hardness and mortality from cardiovascular diseases in several international papers.

In 1975 the Health Council published a report reviewing the chemical, medical and ecological consequences of central water softening. Concerning the medical consequences, the Council concluded that, on basis of possible risks for public health, plans for central water softening should be abandoned for the time being. It recommended further research to establish the identity of the "water factor" causing the suggested correlation and to find out possible mechanisms underlying the relation between the "water factor" and cardiovascular diseases. This advice resulted in a series of studies, coordinated by the Work Group "Gezondheidsaspecten van Centrale Ontharding van Leidingwater" (Health Aspects of Central Water Softening). In this Work Group the Interuniversity Reactor Institute (IRI) in Delft was represented by Prof.dr.ir. J.J.M. de Goeij. The Work Group published a final report on its activities in 1982.

The purpose of the present research, which was initiated by Prof.ir. J.P.W. Houtman of the IRI, was to approach some fundamental aspects of the medical consequences of central water softening by means of animal experiments using radioisotopes. The IRI provided good facilities for such research because of the presence of knowledge, techniques and equipment for radioisotope applications and a facility for production of special (short-living) radioisotopes by the nuclear reactor of the Institute. In the Nuclear Biotechnique Section of the Department of Radiochemistry, where the study was performed, extensive experience in trace element research with laboratory animals was available, particularly in the person of Dr C.J.A. Van den Hamer, the Senior Scientist directly attending the study.

Because of the possible importance of the study with respect to public health aspects, the study was supported by a grant (R712) from the Ministry of Welfare, Health and Cultural Affairs (at that time Ministry of Public Health and Environmental Hygiene). The study was supervized by Prof.ir J.P.W. Houtman of the IRI and a Committee, consisting of Dr T Trouwborst, Drs. J.L.L. Pieters and Dr C.H. Huisman of the Ministries of VROM (Housing, Physical Planning and Environmental Management) and WVC (Welfare, Health and Cultural Affairs) and Drs. E.I. Krajnc of the Rijks Instituut voor de Volksgezondheid (Health Organization Holland).

CHAPTER 1

GENERAL INTRODUCTION

1.1. Outline of the problem.

In 1957 Kobayashi [1] reported a correlation between mortality from apoplexy and the acidity of river water. This finding stimulated Schroeder to compare data on mortality from cardiovascular diseases (CVD) and drinking water quality in the USA. In 1960 he reported a significant negative correlation between drinking water hardness and mortality from CVD [2], which was later on confirmed in many epidemiological studies. However, also conflicting findings were reported. In 1973 Punsar gave an excellent review of the subject [3], from which it became clear that, although a "water factor" seemed to exist, no definite conclusion could be drawn about the character of such a factor. At the end of the 1970s the subject was again reviewed by Neri and Johansen [4] and by Sharrett [5], who emphasized the protective role of Mg in drinking water due to its significant contribution to daily Mg intake. The results of the elaborate British Regional Heart Study, reported in 1980, confirmed the existence of a negative correlation between water hardness and mortality from CVD.

In 1980 the subject was extensively discussed during an international conference on Drinking Water and Cardiovascular Disease, held in the USA, but no clear vision on the subject could be presented [6]. A reason for the complexity of the problem could be that many factors related to water hardness could play a role, either alone or in combination with each other. A list of possible factors underlying the correlation between water hardness and CVD, as can be derived from the literature, is given below:

1. Intake of Ca or Mg from hard drinking water and from foods prepared with hard water (e.g., by cooking) could contribute significantly to dietary Ca or Mg intake and alleviate deficiency, when the diet is sub-adequate in one of these minerals.
2. Soft water could, because of its supposedly higher corrosiveness, release toxic metals from the piping and promote the intake of toxic metals from drinking water and from foods prepared with drinking water.
3. Ca or Mg could protect against uptake of toxic metals from drinking water or from food in the gastrointestinal tract or otherwise influence trace element metabolism in a protective way.
4. During the chemical process of water softening the Na content of the drinking water may be raised, which could promote hypertension.
5. The correlation between water hardness and CVD could be indirect and mediated by another factor (climate, socio-economic factors, etcetera).

Most of these hypotheses should primarily be tested by epidemiological research. A number of epidemiological studies has been carried out in the Netherlands, coordinated by the Dutch Work Group "Health Aspects of Central Water Softening" and has been reported recently [7]. Moreover, an elaborate study of the trace element content of autopsy materials in relation to the frequency and severity of atherosclerotic lesions [8] is in press. In this study drinking water hardness was one of the investigated parameters.

The possible protection of Ca or Mg in drinking water against the uptake of toxic elements from water and food lends itself particularly to fundamental laboratory research. The possibility of interaction between Ca or Mg and trace elements (abbr. TE's) in the gastrointestinal tract can be tested in laboratory animals. Animal studies provide the possibility of carrying out experiments which are to a large extent, if not entirely, impossible in man, e.g., radiotracer experiments using large numbers of animals and experiments in which the animals are sacrificed.

This fundamental approach, viz., the study of the influence of Ca and Mg on TE absorption in animals, covers only one aspect of the softening problematic. Nevertheless, it was considered of importance to get basic information that could help to support or reject hypotheses, formulated on basis of epidemiological research. It could be of value for insight not only in health effects of drinking water softening, but also in general nutrition problems.

1.2. Purpose of the study.

It was the purpose of the present study:

(1) to develop radiotracer methods for investigation of TE metabolism in laboratory animals, particularly for investigation of the influence of Ca and Mg on TE absorption from the gastrointestinal tract and on TE excretion from the body;

(2) to apply this method to a selection of TE's relevant to the question of the negative correlation between drinking water hardness and cardiovascular diseases in order to evaluate the importance of the observed effects with respect to public health;

(3) to gather "spin-off" information important for knowledge of TE metabolism in relation to general nutrition.

Almost exclusively young female Swiss Random mice were used in the studies. Mice were preferred above rats, because large numbers of mice, necessary for statistical evaluation of results, are easier to maintain and handle than large numbers of rats.

1.3. Selection of seven trace elements relevant for investigation.

Seven TE's were selected for investigation of the influence of Ca and Mg on their metabolism, viz., Zn, Cu, Mn, Co, Pb and Cd in the cationic form and Se in the anionic (SeO_3^{2-}) form. Four criteria were used for this selection :

1.3.1. The probability of an interaction with Ca or Mg.

The probability of an interaction of a TE with Ca or Mg is determined by the chemical properties of the TE with respect to those of Ca and Mg and by the mode of interaction considered. If a competition for a common step in the absorption mechanism in the gut is assumed, then a high degree of chemical similarity between the TE and Ca^{2+} or Mg^{2+} will increase the probability of interaction. Cationic metals with a moderate ionic radius, participating in the absorption process in the divalent form, are considered possible competitors with regard to Ca^{2+} and Mg^{2+} : Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, all belonging to the same horizontal row in the periodic system as Ca^{2+} (Fig. 1).

PERIODIC TABLE OF THE ELEMENTS

1 H																2 He	K
3 Li	4 Be											5 B	6 C	7 N	8 O	9 F	10 Ne	L
11 Na	12 Mg											13 Al	14 Si	15 P	16 S	17 Cl	18 Ar	M
19 K	20 Ca	21 Sc	22 Ti	23 V	24 Cr	25 Mn	26 Fe	27 Co	28 Ni	29 Cu	30 Zn	31 Ga	32 Ge	33 As	34 Se	35 Br	36 Kr	N
37 Rb	38 Sr	39 Y	40 Zr	41 Nb	42 Mo	43 Tc	44 Ru	45 Rh	46 Pd	47 Ag	48 Cd	49 In	50 Sn	51 Sb	52 Te	53 I	54 Xe	O
55 Cs	56 Ba	57 La	72 Hf	73 Ta	74 W	75 Re	76 Os	77 Ir	78 Pt	79 Au	80 Hg	81 Tl	82 Pb	83 Bi	84 Po	85 At	86 Rn	P
87 Fr	88 Ra	89 Ac																Q

Fig. 1 Periodic table of the elements.

If the formation of complexes of the TE with Ca^{2+} or Mg^{2+} and food components is assumed, then the probability of formation and the availability for absorption of such complexes are important. Insoluble complexes of metals, e.g., such as the metals mentioned above, but also of others with higher atomic number, e.g., Cd^{2+} and Pb^{2+}, could be formed with Ca^{2+} or Mg^{2+} and phosphate compounds in the food, particularly phytate. Such formation could occur during food processing (cooking), but also during digestion in the gut.

1.3.2. Existence of literature data on interactions with Ca or Mg.

The literature data concerning animal studies on interactions between Ca or Mg and TE's can be divided into three main classes:

α. Direct competition for common steps in the absorption mechanism.

β. Formation of complexes with a low availability for absorption.

γ. Indirect effects through homeostatic adjustment to changes in dietary Ca or Mg intake.

Table 1 summarizes the literature about interactions between Ca and the selected TE's according to the formulated distinction. As concerned Mg interactions with Pb have been reported only (class β_1 [26-28] and γ [29]).

Table 1 Reported interactions between Ca and seven TE's with respect to their absorption from the gut.

TE	Class			
	α	β_1	β_2	γ
Zn	+ [9]	+ [10,11]	+ [12]	+ [10]
Cu	-	-	+ [13]	-
Mn	-	+ [14]	+ [13]	+ [15]
Co	-	-	-	-
Pb	+ [16,17]	+ [18]	+ [19]	+ [20-22]
Cd	+ [23]	-	+ [24]	+ [25]
Se	-	-	-	-

α) Direct interaction between Ca and TE.

β_1) Interaction mediated by phosphate in food.

β_2) Interaction mediated by phytate in food.

γ) Indirect interaction through homeostatic adjustment to changed Ca intake.

+) Interaction reported.

-) No interaction reported.

The direct competition between Ca or Mg and TE's (class α), the formation of complexes with components of food (without phytate; class β_1) and the indirect effects of homeostatic adjustment to changes in dietary Ca or Mg intake (class γ) have been the focus of the studies described in Section 5.2 (Zn, Cu, Co, Se and Cd), 5.3 (Mn) and 5.4 (Pb and Cd). In these Sections more detailed information is given on literature data and on possible mechanisms of interactions. The interaction of TE's with phytate from the food (class β_2) has been investigated extensively by others. These studies are reviewed in Appendix I.

1.3.3. The probability of a role in the etiology of CVD.

Many TE are mentioned, in one way or another, in relation to cardiovascular disease [30-32]. Some, however, recur more often and with greater emphasis in literature than others. Zn and Cu have mainly been mentioned in relation to each other. A low Cu/Zn-ratio in the diet has been related to hypercholesterolemia as a result of Cu-deficiency [33]. Co and Se have been mentioned in relation to defined cases of cardiovascular illness: Co intoxication (in combination with a high ethanol and low protein intake) as the causative factor of cardiomyopathy in heavy beer drinkers [34] and Se-deficiency as the causative factor of cardiomyopathy in children and young women living in the Keshan regio in China [35] and in a patient during total parenteral nutrition [36]. Mn has been related to the "sudden death" category of cardiovascular disease through its possible competitive interaction in the heart with Mg, an important stabilizer of the heart rhythm [37]. Finally, the toxic heavy metals Pb and Cd have been mentioned as inductive agents of hypertension and atherosclerosis [38,39].

1.3.4. The availability of a suitable radioisotope.

The study of TE metabolism with the aid of radiotracers involves measurement of the whole-body retention of the tracer during several days. Therefore, a radioactive isotope of the relevant TE should be available, that meets the requirement of being a γ-emitter with a physical half-life ($t_{1/2}$) of at least a few days. The γ-energy is restricted to a certain energy range so that the emitted intensity from the mouse and absorption by the detector are high enough to avoid extreme quantities of radioactivity necessary to obtain sufficient signal. Table 2 lists the radioisotopes used, together with their physical half-lifes and γ-energies.

Table 2 List of radioisotopes used in the metabolic experiments.

Radioisotope	$t_{1/2}$	E_γ (MeV)
^{65}Zn	245 d	1.115, 0.511 (annihilation)
^{64}Cu	12.8 h	0.511 (annihilation)
^{54}Mn	303 d	0.835
^{60}Co	5.26 y	1.173, 1.332
^{203}Pb	52.1 h	0.28, 0.40
^{115m}Cd	53.5 h	0.49, 0.53
^{75}Se	120 d	0.12, 0.14, 0.2, 0.28

The measurements of the short-living radioisotopes (^{64}Cu, ^{203}Pb, ^{115m}Cd) required correction for radioactive decay. The short half-life of ^{64}Cu (12.8 h) did not allow measurement of the body retention during more than 3 days. However, the longer living ^{67}Cu (61.9 h) was not available at the time of experimentation. Correction for radioactive decay over periods of days, for instability of counting equipment and for geometric differences between counters was done by simultaneous measurement of standard solutions of the radioisotope concerned.

Literature cited.

1. Kobayashi, J. (1957) On geographical correlation between the chemical nature of river water and death-rate from apoplexy. Ber. Ohara Inst. landw. Biol. 11, 12-21.
2. Schroeder, H.A. (1960) Relation between mortality from cardiovascular disease and treated water supplies. J. Am. Med. Assoc. 172, 1902-1908.
3. Punsar, S. (1973) Cardiovascular mortality and quality of drinking water. An evaluation of the literature from an epidemiological point of view. Work-environm.-hlth 10, 107-125.
4. Neri, L.C. & Johansen, H.L. (1978) Water hardness and cardiovascular mortality. Ann. N.Y. Acad. Sci. 304, 203-219.
5. Sharrett, A.R. (1977) Water hardness and cardiovascular disease - elements in water and human tissues. Sci. Total Environ. 7, 217-226.
6. Proc. Int. Conf. "Drinking Water and Cardiovascular Disease" (1980) J. Environm. Pathol. Toxicol. 4, 5-325.
7. Rapport van de Werkgroep "Gezondheidsaspecten van Centrale Ontharding van Leidingwater" (1982).
8. Aalbers, T.G. (1984) Thesis, Delft (in press).
9. Adham, N.F. & Song, M.K. (1980) Effect of calcium and copper on zinc absorption in the rat. Nutr. Metab. 24, 281-290.
10. Heth, D.A., Becker, W.M. & Hoekstra, W.G. (1966) Effect of calcium, phosphorus and zinc on zinc-65 absorption and turnover in rats fed semipurified diets. J. Nutr. 88, 331-337.
11. Cabell, C.A. & Earle, I.P. (1965) Additive effect of calcium and phosphorus on utilization of dietary zinc. J. Animal Sci. 24, 800-804.
12. Van Barneveld, A.A. (1983) De invloed van fytaat in de voeding: een overzicht. Voeding 44, 51-58 (see Appendix I).
13. Davies, N.T. & Nightingale, R. (1975) The effects of phytate on intestinal absorption and secretion of zinc, and whole body retention of Zn, copper, iron and manganese in rats. Br. J. Nutr. 34, 243-258.
14. Leach, R.M. & Lilburn, M.S. (1978) Manganese metabolism and its function. Wld Rev. Nutr. Diet. 32, 123-134.
15. Lassiter, J.W., Miller, W.J., Pate, F.M. & Gentry, R.P. (1972) Effect of

dietary calcium and phosphorus on ^{54}Mn metabolism following single tracer intraperitoneal and oral doses in rats. Proc. Soc. exp. Biol. Med. 139, 345-348.

16. Meredith, P.A., Moore, M.R. & Goldberg, A. (1977) The effect of calcium on lead absorption in rats. Biochem. J. 166, 531-537.
17. Barton, J.C., Conrad, M.E., Harrison, L. & Nuby, S. (1978) Effects of calcium on the absorption and the retention of lead. J. Lab. Clin. Med. 91, 366-376.
18. Quarterman, J., Morrison, J.N. & Carey, L.F. (1974) The influence of dietary calcium and phosphate on lead metabolism. Tr. Subst. Environ. Hlth 7, 347-352.
19. Wise, A. (1981) Protective action of calcium phytate against acute lead toxicity in mice. Bull. Environm. Contam. Toxicol. 27, 630-633.
20. Goyer, R.A. (1978) Calcium and lead interactions: some new insights. J. Lab. Clin. Med. 91, 363-365.
21. Mahaffey, K.R. (1980) Nutrient-lead interactions. In: Lead toxicity, pp. 425-460, R.L. Singhal & J.A. Thomas, eds, Urban and Schwarzenberg, Baltimore.
22. Chisolm, Jr., J.J. (1980) Lead and other metals: a hypothesis of interaction. In: Lead toxicity, pp. 461-482, R.L. Singhal & J.A. Thomas, eds, Urban and Schwarzenberg, Baltimore.
23. Koo, S.I., Fullmer, C.S. & Wasserman, R.H. (1978) Intestinal absorption and retention of ^{109}Cd: effects of cholecalciferol, calcium status and other variables. J. Nutr. 108, 1812-1822.
24. Wise, A. & Gilburt, D.J. (1981) Binding of cadmium and lead to the calcium-phytate complex in vitro. Toxicol. Lett. 9, 45-50.
25. Bremner, I. (1978) Cadmium toxicity. Nutritional influences and the role of metallothionein. Wld Rev. Nutr. Diet. 32, 165-197.
26. Fine, B.P., Barth, A., Sheffet, A. & Lavenhar, M.A. (1976) Influence of magnesium on the intestinal absorption of lead. Environ. Res. 12, 224-227.
27. Singh, N.P., Thind, I.S., Vitale, L.F. & Pawlow, M. (1979) Intake of magnesium and toxicity of lead: an experimental model. Arch. Environ. Hlth 34, 168-172.
28. Krall, A.R. & McLean, M.S., Jr. (1980) Effects of varying dietary magnesium and phosphate on tissue lead content of lead-fed rats. In: Magnesium in health and disease, pp. 955-960, M. Cantin and M.S. Seelig, eds., Spectrum, New York, USA.
29. Cerklewski, F.L. (1983) Influence of maternal magnesium deficiency on tissue lead content of rats. J. Nutr. 113, 1443-1447.
30. Masironi, R. (1974) Trace elements in relation to cardiovascular diseases. WHO Offset Publication no.5, pp. 1-45, World Health Organization, Geneva.

31. Schroeder, H.A. (1974) The role of trace elements in cardiovascular diseases. Med. Clin. N. Am. 58, 381-396.
32. Mertz, W. (1982) Trace minerals and atherosclerosis. Fed. Proc. 41, 2807-2812.
33. Klevay, L.M. (1973) Hypercholesterolemia in rats produced by an increase in the ratio of zinc to copper ingested. Am. J. Clin. Nutr 26, 1060-1068.
34 Alexander, C.S. (1969) Cobalt and the heart. Ann. Int Med. 70, 411-413.
35. Anonymous (1980) Prevention of Keshan cardiomyopathy by sodium selenite. Nutr. Rev. 38, 278-279.
36. Johnson, R.A. et al. (1981) An occidental case of cardiomyopathy and selenium deficiency. N. Engl. J. Med. 304, 1210-1212.
37. Marienfeld, C.J. & Collins, M. (1981) The ebb and flow of manganese. a possible pathogenic factor in birth defects, cancer and heart disease. Tr. Subst Environm. Hlth 15, 3-20.
38. Perry, Jr., H.M. (1976) Review of hypertension induced in animals by chronic ingestion of cadmium. In: Trace elements in human health and disease, II. Essential and toxic elements, pp. 417-430, A.S. Prasad and D. Oberleas, eds, Academic Press, New York, 1976.
39. Revis, N.W., Zinsmeister, A.R. & Bull, R (1981) Atherosclerosis and hypertension induction by lead and cadmium ions: an effect prevented by calcium ion. Proc. Natl. Acad. Sci. USA 78, 6494-6498.

CHAPTER 2

METABOLISM OF SEVEN TRACE ELEMENTS

This Chapter gives a short description of the metabolism of the seven trace elements selected for investigation, viz., Zn, Cu, Mn, Co, Pb, Cd and Se.

2.1. Zinc.

Zinc is mainly absorbed in the upper intestine. The absorption is probably mediated by low-molecular-weight Zn binding ligands in the lumen, originating from the pancreatic or biliary juice. These ligands could transfer Zn from the food to the mucosal receptors or even transport it into the mucosal cell. In this cell Zn is bound to proteins of high and low molecular weight. An important Zn binding protein is metallothionein, a protein of 7000 Daltons to which a regulatory function is attributed. After absorption into the circulation Zn is transported primarily to the liver and the pancreas. The pancreas synthesizes digestive enzymes which contain Zn. By secretion of these enzymes in the pancreatic digestive juice part of the absorbed Zn is excreted again into the lumen. When the Zn intake is higher than the daily need, the excess of Zn is removed by endogenous excretion. It is not known, whether this Zn is excreted with the pancreatic juice, bound to enzymes or not, with the bile or with other intestinal secretions. It is also not known, whether this excreted Zn could be reabsorbed again. Reabsorption has been assumed in several ^{65}Zn absorption studies in which the results were explained by isotope dilution in the gut (see also Appendix II).

Zn excretion is not the only way of homeostatic regulation; also absorption is regulated. Therefore, endogenous Zn excretion serves as a secondary adjustment of the amount of Zn retained in the body. The excretion of Zn in the urine is very low as compared to faecal excretion. In man excretion in sweat seems to contribute significantly to the total excretion.

List of relevant literature.

1. Becker, W.M. & Hoekstra, W.G. (1971) The intestinal absorption of zinc. In: Intestinal absorption of metal ions, trace elements and radionuclides, pp. 229-256, S.C. Skoryna and D. Waldron-Edward, eds, Pergamon Press, Oxford, England.
2. Halsted, J.A., Smith, Jr., J.C. & Erwin, M.I. (1974) A conspectus of research on zinc requirements of man. J. Nutr. 104, 345-378.
3. Evans, G.W. (1976) Zinc absorption and transport. In: Trace elements in human health and disease, I. Zinc and copper, pp. 181-187, A.S. Prasad and D. Oberleas, eds, Academic Press, New York, USA.

4. Underwood, E. (1977) Zinc. In: Trace elements in human and animal nutrition, 4th ed., pp. 196-242. Academic Press, New York, USA.
5. Cousins, R.J. (1979) Regulation of zinc absorption: role of intracellular ligands. Am. J. Clin. Nutr. 32, 339-345.
6. Solomons, N.W. (1982) Biological availability of zinc in humans. Am. J. Clin. Nutr. 35, 1048-1075.
7. DiSilvestro, R.A. & Cousins, R.J. (1983) Physiological ligands for copper and zinc. Ann. Rev. Nutr. 3, 261-288.

2.2. Copper.

Cu is mainly absorbed from the stomach and upper small intestine. Its absorption depends - among others - on the dietary intake and on the chemical form of Cu. Cu^{2+} is the form which is transferred through the mucosa; Cu^{1+} is poorly absorbed. Ascorbic acid reduces Cu absorption, because it reduces Cu^{2+} to Cu^{1+}.

At least two mechanisms seem to operate in Cu absorption, one involving Cu binding to amino acids, which are absorbed as Cu complexes, and one involving Cu binding to proteins, which transfer Cu to receptors on the mucosal membrane. In the mucosal cell Cu is bound to other Cu proteins, one of which (metallothionein) may serve as a storage depot and simultaneously may have a protective and regulatory function.

The important role of metallothionein both in Cu and in Zn absorption suggests that these two metals may interact at the absorption level. Indeed, Zn seems to reduce the absorption of Cu, when given in high amounts several hours before Cu administration. It is suggested that Zn induces the synthesis of metallothionein in the mucosal cell, which traps the incoming Cu and stores it until desquamation of the cell, thus preventing transfer of Cu to the circulation. This effect of Zn on Cu absorption is used in the treatment of Wilson's disease, a defect in Cu excretion, to induce a negative Cu balance.

The absorbed Cu is bound to albumin and amino acids in the plasma and transported to liver and kidneys, which have a large binding capacity for this metal. In the liver Cu is incorporated into caeruloplasmin and as such released again into the circulation. Superfluous Cu is excreted with bile. Biliary Cu seems to be bound in complexes, which cannot be reabsorbed, so endogenous circulation of Cu probably is negligible. Only a small fraction of absorbed Cu is excreted with urine.

List of relevant literature.

1. Van Campen, D R. (1971) Absorption of copper from the gastrointestinal tract. In: Intestinal absorption of metal ions, trace elements and radionuclides, pp. 211-227, S C. Skoryna and D Waldron-Edward, eds, Pergamon Press, Oxford, England.
2. Underwood, E. (1977) Copper In: Trace elements in human and animal nutrition, 4th ed., pp. 56-108, Academic Press, New York, USA
3. Mason, K.E. (1979) A conspectus of research on copper metabolism and requirements of man. J. Nutr. 109, 1979-2066.
4. Solomons, N.W (1981) Zinc and copper in human nutrition. In: Nutrition in the 1980s. Constraints on our knowledge, pp 97-127, Alan R Liss, Inc., New York, USA.
5. Sandstead, H.H. (1982) Copper bioavailability and requirements Am J. Clin. Nutr. 35, 809-814.
6. DiSilvestro, R.A. & Cousins, R.J. (1983) Physiological ligands for copper and zinc. Ann. Rev Nutr. 3, 261-288

2.3. Manganese.

Mn is absorbed throughout the gastrointestinal tract, but the total absorption seems to be low. The absorption process shows two-step kinetics, one step being a binding of Mn to mucosal receptors and the next step being transfer of Mn to the serosal side and release into the circulation. The absorption of Mn appears to be strongly related to that of Fe. This is suggested on the one hand by a competition between Mn and Fe for both steps of the absorption mechanisms of the metals and on the other hand by similar increases of Mn and Fe absorption in Fe-deficient animals.

In contrast to the absorption, the metabolism of Mn in the body, i.e., after absorption from the gut, seems to be very specific The only TE that can influence turnover of ^{54}Mn seems Mn itself. The homeostasis of Mn is mainly regulated by the biliary excretion in the faeces The liver plays an important role by very efficiently picking up absorbed Mn^{2+} from the circulation. A small part is oxidized to Mn^{3+} and released again into the circulation, where it is transported to mitochondria-rich tissues The remaining Mn^{3+} not needed for the metabolism is excreted with bile When the capacity of the liver to remove Mn is surpassed, excretion may occur with pancreatic juice and through the intestinal wall. Urinary excretion of Mn is negligible. The possibility of reabsorption of excreted Mn (endogenous circulation) has not been investigated.

List of relevant literature.

1. Leach, Jr., R.M. (1976) Metabolism and function of manganese. In: Trace elements in human health and disease, II. Essential and toxic elements, pp. 235-247, A.S. Prasad and D. Oberleas, eds, Academic Press, New York, USA.
2. Underwood, E. (1977) Manganese. In: Trace elements in human and animal nutrition, 4th ed., pp. 170-195, Academic Press, New York, USA.
3. Leach, R.M. & Lilburn, M.S. (1978) Manganese metabolism and its function. Wld Rev. Nutr. Diet. 32, 123-134.
4. Thomson, A.B.R., Olatunbosun, D. & Valberg, L.S. (1971) Interrelation of intestinal transport system for manganese and iron. J. Lab. Clin. Med. 78, 642-655.

2.4. Cobalt.

Little is known about the metabolism of Co. Inorganic Co is probably not an essential element. Incorporated in vitamin B_{12} Co plays an essential role, e.g., in erythropoiesis. Mammals are not capable of vitamin B_{12} synthesis. In rodents dietary Co can alleviate vitamin B_{12} deficiency, because the intestinal microflora of the colon can synthesize vitamin B_{12} with inorganic Co and thus vitamin may be absorbed and utilized. In man absorption of vitamin B_{12} from the colon does not occur, so food should supply adequate amounts of vitamin B_{12}.

Absorption of inorganic cobalt shows strong similarity with absorption of Fe and of Mn. With both elements it competes for uptake into the mucosal cell and for transfer to the circulation; its absorption is similarly increased in Fe-deficient animals. Apparently these three metals are absorbed through very similar absorption pathways or even have some steps in common.

The similarity of Mn, Fe and Co absorption could be explained by the chemical relation between the three metals. In the periodic table of elements (page 3) these metals are situated next to each other, having practically the same ionic radius.

In contrast to Mn, Co is mainly excreted in the urine and to a smaller extent in the faeces, probably with the bile, but not with pancreatic juice. Additional losses may occur in sweat and hair.

List of relevant literature.

1. Valberg, L.S. (1971) Cobalt absorption. In: Intestinal absorption of metal ions, trace elements and radionuclides, pp. 257-263, S.C. Skoryna and D. Waldron-Edward, eds, Pergamon Press, Oxford, England.

2. Underwood, E. (1977) Cobalt. In: Trace elements in human and animal nutrition, 4th ed., pp. 132-158, Academic Press, New York, USA.
3. Thomson, A.B.R. & Valberg, L.S. (1972) Intestinal uptake of iron, cobalt, and manganese in the iron-deficient rat. Am. J. Physiol. 223, 1327-1329.
4. Flanagan, P.R., Haist, J. & Valberg, L.S. (1980) Comparative effects of iron deficiency induced by bleeding and a low-iron diet on the intestinal absorptive interactions of iron, cobalt, manganese, zinc, lead and cadmium. J. Nutr. 110, 1754-1763.

2.5. Lead.

Although Pb in very small amounts has been reported to be an essential element to animals, Pb is considered a very toxic metal. Generally it is poorly absorbed from the gastrointestinal tract. The protective mechanism against Pb absorption seems to be developed during infancy, so children may exhibit a much higher absorption than adults. Pb is a very large cation as compared to most essential trace elements. Pb absorption by substitution for these metals in their absorption pathways seems therefore less likely. It is suggested, that Pb is not actively transported through the mucosa into the circulation, but passes the mucosa by leakage through the desmosomes and tight junctions, which keep the mucosal cells together, or through the tip of the intestinal villi on the site where the old mucosal cells are repelled and the closed structure of the villus is interrupted.

After absorption a small part of Pb is incorporated in bone and kidneys. Most of the absorbed Pb is excreted, partly with bile and partly with urine. Pb primarily affects haem synthesis by inhibition of several enzymes involved. Furthermore it has an adverse influence on the central nervous system, which seems to be related to neurotransmission processes.

List of relevant literature.

1. Schwarz, K. (1974) New essential trace elements (Sn, V, F, Si): progress report and outlook. TEMA-2, pp. 355-380, W.G. Hoekstra, Suttie, J.W., Ganther, H.E. and Mertz, W., eds.
2. Smith, J.L. (1976) Metabolism and toxicity of lead. In: Trace elements in human health and disease, II. Essential and toxic elements, pp. 443-452, A.S. Prasad and D. Oberleas, eds, Academic Press, New York, USA.
3. Underwood, E. (1977) Lead. In: Trace elements in human and animal nutrition, 4th ed., pp. 410-423, Academic Press, New York, USA.
4. Mahaffey, K.R. (1980) Nutrient-lead interactions. In: Lead toxicity, pp. 425-460, R.L. Singhal & J.A. Thomas, eds, Urban and Schwarzenberg, Baltimore.

5 Chisolm, Jr., J.J. (1980) Lead and other metals: a hypothesis of interaction. In. Lead toxicity, pp. 461-482, R.L. Singhal & J.A. Thomas, eds, Urban and Schwarzenberg, Baltimore.

2.6. Cadmium.

Cd has, just as Pb, been reported to be essential to animals in very small amounts, but is primarily considered a toxic metal. It is poorly absorbed from the gastrointestinal tract and possibly passes the mucosa by non-Cd-specific pathways, e.g., that of Zn. The body seems to protect itself against Cd in two ways. Firstly, when dietary Cd intake is high, the synthesis of a Cd binding protein (metallothionein) is induced in the mucosa. This metallothionein seems to capture the incoming Cd and make it unavailable for uptake into the circulation. The Cd-thionein binding is preserved until repulsion of the old mucosal cell, after which Cd is removed in the faeces. Secondly, Cd also induces the synthesis of metallothionein in other organs like liver and kidneys. Absorbed Cd is transported to these organs and bound to metallothionein which has a detoxifying function, because thionein-bound Cd is considered to be harmless and does not damage the cell.

The Cd bound in the kidney is released at a very slow rate; its half-life is many years. Therefore, Cd accumulates in the kidneys during lifetime and the kidney content gradually increases with age. As long as this Cd content is below a critical level (200 µg/g wet weight), it remains bound to metallothionein and no kidney damage seems to occur. Above this critical level the detoxifying capacity of the kidneys is surpassed and severe kidney damage may occur, reflected - among others - in proteinuria.

Symptoms of Cd intoxication are not limited to cases in which the critical kidney level is surpassed. Symptomatic Cd intoxication may also occur, when a high Cd intake is combined with a low Ca or vitamin D intake. Cd interferes with bone mineralization, which is strongly accentuated by a relative Ca-deficiency, as may occur in mothers after many pregnancies. The resulting Itai-itai disease (bone malformation) was a great public health problem in Japan some decades ago. In laboratory animals also a hypertensive effect of chronic low Cd doses has been found. This subject, however, is controversial.

List of relevant literature.

1. Schwarz, K. & Spallholz, J. (1976) Growth effects of small cadmium supplements in rats. Fed. Proc. 35, 255 (abstract)
2. Fox, M.R.S. (1976) Cadmium metabolism - a review of aspects pertinent to evaluating dietary cadmium intake by man. In. Trace elements in human

health and disease, II. Essential and toxic elements, pp. 401-416, A.S. Prasad and D. Oberleas, eds, Academic Press, New York, USA.
3. Squibb, K.S., Cousins, R.J., Silbon, B.L. & Levin, S. (1976) Liver and intestinal metallothionein: function in acute cadmium toxicity. Expl. molec. Pathol. 25, 163-171.
4. Underwood, E. (1977) Cadmium. In: Trace elements in human and animal nutrition, 4th ed., pp. 243-257, Academic Press, New York, USA.
5. Bremner, I. (1978) Cadmium toxicity. Nutritional influences and the role of metallothionein. Wld Rev. Nutr. Diet. 32, 165-197.
6. Holt, D. & Webb, M. (1983) Intestinal and hepatic binding of cadmium in the neonatal rat. Arch. Toxicol. 52, 291-301.

2.7. Selenium.

Se occupies a special place in the set of trace elements selected in this study, because it is the only element investigated in its anionic form, viz., as selenite (SeO_3^{2-}). Selenite is very well absorbed from the gut (over 90% in rats). After entrance into the circulation it is probably reduced to selenide by the erythrocyte before being bound to plasma proteins (mainly β-lipoproteins). From the plasma it is primarily taken up in the liver and kidneys. In the tissues Se is incorporated in several types of proteins, e.g., glutathion peroxidase.

Homeostasis of Se is achieved by urinary excretion of Se as trimethyl selenonium ion and a substance called U-2. At high doses of Se some Se may be excreted by exhalation as dimethyl selenide. The faecal endogenous excretion originates from biliary, pancreatic and intestinal secretions and does not vary with Se intake.

Se has a strong tendency to form complexes with heavy metals. This may explain the detoxifying action of Se against mercury and cadmium. At higher intake Se may be strongly carcinogenic.

List of relevant literature.

1. Burk, R.F. (1976) Selenium in man. In: Trace elements in human health and disease, II. Essential and toxic elements, pp. 105-133, A.S. Prasad and D. Oberleas, eds, Academic Press, New York, USA.
2. Underwood, E. (1977) Selenium. In: Trace elements in human and animal nutrition, 4th ed., pp. 302-346, Academic Press, New York, USA.
3. Young, V.R., Nahapetian, A. & Janghorbani, M. (1982) Selenium bioavailability with reference to human nutrition. Am. J. Clin. Nutr. 35, 1076-1088.

CHAPTER 3

TRACE ELEMENT METABOLISM : MODELS AND CONCEPTS

This Chapter gives a review of three important models for investigation of trace element metabolism, one of which is adopted for the present study.

3.1. The "utilization" model of Weigand and Kirchgessner (W&K).

The dynamic factorial model on trace element utilization of Weigand and Kirchgessner [1,2], further referred to as "utilization" model, describes the coming in and going out of streams of TE's in an organism (man, animal) during a balance period in which the conditions do not change. The TE status (the condition of an organism with respect to its TE needs), TE utilization (the use of TE's for maintenance, growth and production) and TE homeostatic control (the tendency of an organism to keep its TE status at an optimum level) play a central role in this model.

Table 1 Parameters of the "utilization" model of W&K.

Symbol	Term	Definition
I	Intake	
F	Faecal excretion	$F = F_i + F_e$
F_i	Unabsorbed fraction of intake	
F_e	Endogenous excretion	$F_e = F_{em} + F_{eh}$ [1]
U	Urinary excretion	$U = U_m + U_h$ [1]
V	Various losses	$V = V_m + V_h$ [1]
D	Apparent digestion	$D = I - F$
A	Actual absorption	$A = I - F_i$
R	Apparent retention	$R = I - F - U - V$
T	True retention	$T = A - F_{eh} - U_h$
Q	Utilization	$Q = Q_m + Q_p$
Q_m	Utilization for maintenance	$Q_m = F_{em} + U_m + V$
Q_p	Utilization for production	$Q_p = R - \Delta R$
ΔR	Non-utilized deposition	$\Delta R = R - Q_p$
E	Endogenous losses	$E = A - Q$

[1] F_{em}, U_m and V_m are indispensible components of excretion due to maintenance; F_{eh}, U_h and V_h are facultative components of excretion due to homeostatic control (V_h is generally neglected).

The parameters of the "utilization" model are summarized in Table 1. To explain the meaning of these parameters, in the following text the extra parameter S (sum of excretory products) is introduced, which is not used in the original model. It should be emphasized, however, that all other parameters are defined by Weigand and Kirchgessner.

Trace element metabolism in a living organism can be defined as a process, in which a trace element is taken up from the environment, utilized and, dependent on the mode of utilization, retained or excreted. In a first approach to this process the organism can be seen as a closed system, described by equation (1):

$$I = R + S \qquad (1)$$

in which I = intake
R = retention
S = sum of excretory products

Generally the organism (man, mouse, rat) excretes TE's in faeces and urine and also looses some TE through other pathways, so we can define S as:

$$S = F + U + V \qquad (2)$$

in which F = faecal excretion
U = urinary excretion
V = various losses (see Note 1)

Combination of (1) and (2) shows that:

$$R = I - F - U - V \qquad (3)$$

Sometimes a distinction is made between faecal excretion F on the one hand and urinary excretion U and various losses V on the other hand. Then U and V are considered as digestion products, in contrast to F which is assumed to consist of not absorbed TE only. This results in equation (4) and (4a)

$$D = I - F \qquad (4)$$
$$D = R + U + V \qquad (4a)$$

in which D = digestion or apparent absorption

This concept, shown in Fig. 1, offers the advantage of using parameters (D and R), which can be easily calculated from balance data (I, F and U; V is estimated or neglected).

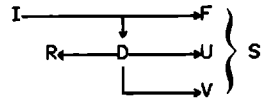


Fig. 1 "Digestion" concept in TE metabolism.

The disadvantage of the digestion concept is that it ignores faecal excretion of endogenous origin. In fact faecal excretion F has two distinct components, viz., faecal loss due to incomplete absorption (F_i) and faecal loss due to endogenous excretion (F_e):

$$F = F_i + F_e \qquad (5)$$

As a result the digestion D is replaced by the actual absorption A, which is defined as the amount of TE absorbed from the gastrointestinal tract into the body. As the unabsorbed part of the intake is lost in the faeces, A relates to I as follows:

$$A = I - F_i \qquad (6)$$

The relation between the actual absorption A and the digestion D is shown in equation (7):

$$A = D + F_e \qquad (7)$$

The faecal endogenous excretion F_e can not be measured directly in balance studies, because it cannot be distinguished from unabsorbed TE, unless an isotope is used to tracer the endogenous TE. An isotope dilution method for measuring F_e during a balance trial is developed by Weigand and Kirchgessner [3,4] (see Note 2).

The distinction that has been made for the faecal excretion $F=F_i+F_e$ can be extended to the sum of excretory products $S=F+U+V$. U and V have an endogenous origin, so F_i is the only component originating from unabsorbed TE. Therefore S can be written as:

$$S = F_i + S_e \qquad (8)$$

In which:

$$S_e = F_e + U + V \qquad (9)$$

Combination of (8) with (1) and (6) shows that the retention R is the difference between the amount of actually absorbed TE (A) and the amount of endogenous TE excreted in the faeces (S_e):

$$R = A - S_e \qquad (10)$$

In case of balance between intake and excretion R=0, so $A=S_e$. The body then excretes an amount of endogenous TE equal to the amount absorbed in the gut. The concept on basis of the actual absorption A is shown in Fig. 2.

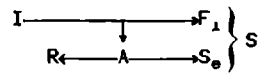


Fig. 2 "Actual absorption" concept in TE metabolism.

When an organism gets far less than its daily requirement of a TE, it will generally excrete a minimum amount of TE as a result of maintenance processes, such as the excretion of TE containing enzymes in digestive juice, the excretion of TE in bile or in urine due to incomplete recovery from the glomerular filtrate and various other losses. This excretion is indispensible and will result in a negative balance. When far more than the daily requirement is absorbed, an important amount of TE will be excreted as a result of homeostatic regulation. Thus we can distinguish two components in S_e , viz., an indispensible component due to maintenance (S_{em}) and a facultative component due to homeostatic control (S_{eh}):

$$S_e = S_{em} + S_{eh} \qquad (11)$$

and because $S_e=F_e+U+V$:

$$F_e = F_{em} + F_{eh} \qquad (11a)$$
$$U = U_m + U_h \qquad (11b)$$
$$V = V_m + V_h \qquad (11c)$$

In the model V_h is neglected, so is $V=V_m$ Further is:

$$S_{em} = F_{em} + U_m + V \qquad (12a)$$
$$S_{eh} = F_{eh} + U_h \qquad (12b)$$

Due to this distinction three new metabolic parameters can be formulated, viz., true retention T, utilization Q and endogenous utilization losses E.

True retention T is defined as the difference between the amount of actually absorbed TE (A) and the amount of superfluous endogenous TE excreted because of homeostatic control (S_{eh})

$$T = A - S_{eh} \qquad (13)$$

The relation between the true retention T and the apparent retention R can be derived from equations (10), (11) and (13):

$$R = T - S_{em} \qquad (14)$$

Utilization Q is defined as the amount of TE, which is utilized partly for maintenance (Q_m) and partly for production of new body mass (Q_p) (growth, reproduction):

$$Q = Q_m + Q_p \qquad (15)$$

Endogenous utilization losses E are defined as the difference between the amount of actually absorbed TE (A) and the amount of TE utilized (Q):

$$E = A - Q \qquad (16)$$

They are excreted because of homeostatic control (S_{eh}), or deposited in the body (ΔR):

$$E = S_{eh} + \Delta R \qquad (17)$$

The body deposit ΔR does not have any metabolic or anabolic function at the time of deposition. At a later stage, however, it could become available again and be utilized as well. Therefore, the term "loss" does not apply very well to this fraction of E.

ΔR is part of the apparent retention R, which further consists of the amount of TE used for production of new body mass (Q_p):

$$R = Q_p + \Delta R \qquad (18)$$

or:

$$Q_p = R - \Delta R \qquad (18a)$$

Q_m is ultimately excreted as S_{em} :

$$Q_m = S_{em} \qquad (19)$$

Combination of (18) and (19) with (15) shows that $Q=S_{em}+R-\Delta R$, so according to (14):

$$Q = T - \Delta R \qquad (20)$$

For a better understanding of the "utilization" model of Weigand and Kirchgessner two schemes are presented (Fig. 3 and Fig. 4), one containing parameters I, A, T, R, Q and S, the other containing more detailed parameters.

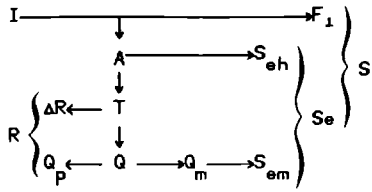


Fig. 3 Course schematic presentation of the "utilization" model.

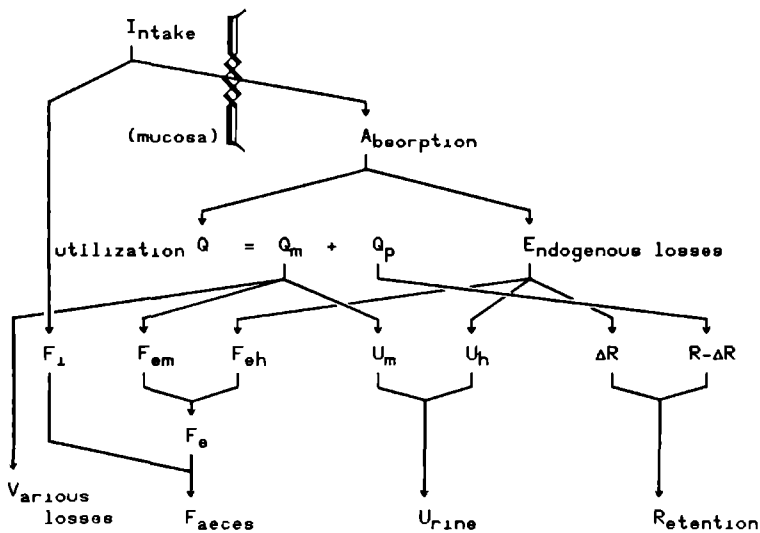


Fig. 4 Detailed schematic presentation of the "utilization" model.

In balance studies the parameters I, F and U are easily measured. V can often be neglected. F_e and F_{em} can be measured by means of the isotope dilution method, F_{em} under conditions of sub-adequate TE intake. When these parameters are known, the parameters $F_\perp$, F_{eh}, A, D and R can be calculated. Q can be measured under conditions of just adequate TE supply (S_{eh}=0 and ΔR=0, so E=0 and Q=A). T can be calculated from (13) and (12b) assuming that U_h=0, so T=A-F_{eh}

Notes:

1) V may have a very divergent character. Some losses are caused by continuous processes (exhalation of volatile compounds, sweat, hair, nails, skin), others may occur occasionally (excessive transpiration, menstrual and donative blood, sperm) or by accidents (blood, loss of intracellular fluid through the skin, e.g. in burns). Some faecal and urinary losses should perhaps be understood as various losses, e.g., desquamation of mucosa and losses caused by medication (chelation therapy) and intestinal disease.

2) Isotope dilution method of Weigand and Kirchgessner for calculation of endogenous excretion, e.g., applied to the TE Zn.
^{65}Zn is injected intramuscularly 9 days before the balance trial. It is assumed that in this period the ^{65}Zn is homogenously distributed over the rapidly exchanging Zn pools in the body, which are the source of endogenous Zn excreted in the intestine, and that uniform labelling of these pools has been achieved. During the balance trial the specific activity of ^{65}Zn in faeces and in plasma or urine can be measured. It is assumed that the specific activity of the excreted endogenous Zn is identical to that of plasma or urine. According to the isotope dilution theory F_e can easily be calculated from the formula:

$$F_e = \frac{\sigma_f}{\sigma_m} \times F \qquad (21)$$

in which: σ_f = specific activity of faeces
σ_m = specific activity of plasma or urine.

When F_e is known, then A, D and F_i can easily be calculated from the balance data. It should be noticed that F_e calculated by the isotope dilution method is the gross faecal endogenous excretion, whereas F_e in equation (5) and (7) is the nett faecal endogenous excretion, the difference being the TE which possibly is absorbed again from the lumen. The use of the measured value of F_e in the balance equations is only valid when the excreted endogenous TE is not absorbed again. If there is a certain degree of absorption of excreted endogenous TE, then the nett F_e is smaller than the gross F_e and as a result F_i will be underestimated and A overestimated.

3.2. The "bioavailability" model.

The bioavailability of a TE can be defined as the extent to which the chemical form of the TE and the composition of its carrier allows absorption from the gastrointestinal tract. One approach for investigation of the bioavailability of a certain form of TE is to limit the dietary supply of the TE to a level below requirement, so that the bioavailability of the TE determines the actual absorption. Because the intake is below the requirement, the bioavailability also determines the rate of those metabolic processes in the body, which depend on the TE in question. The effect of limited quantities of the TE in the form to be investigated on these metabolic processes can then be compared with that of similarly limited quantities of the TE in a specified form of known high availability, for example the sulfate salt of a TE. Bioavailability, measured according to this approach, is always related to a specified standard. Therefore, it is better to speak of relative bioavailability (RBA).

An example of this type of approach is the slope-ratio assay [5,6] for testing of the RBA of Zn (RBAZ) in rats. Weight gain [5] and femur Zn content [6] show a linear relationship, when plotted on a semilogarithmic scale against the (sub-adequate) dietary Zn intake. When such plots are made for sub-adequate levels of dietary Zn sulfate and for sub-adequate levels of dietary Zn in a less available form, the RBAZ of this form, expressed as fraction of the availability of Zn sulfate, can be calculated from the slope-ratio of both curves. Several variants of the slope-ratio assay for determination of the RBAZ were intercompared by Franz et al. [7].

3.3. The "isotope" model.

Another approach for measuring TE availability and metabolism is the use of radioactive or enriched stable isotopes. When a tracered TE is orally administered, the route of this TE through the gastrointestinal tract and, after absorption, through body pools can be followed independent of TE already present or TE taken up after the dose. Because a tracered TE follows the same routes as dietary TE, the same type of parameters can be used as in the "utilization" model. There is, however, an important difference. In the "utilization" model the parameters represent <u>flows</u>, expressed as mass/unit of time. In the "isotope" model the parameters represent <u>quantities</u>, expressed as radioactivity, or more convenient, <u>fractions</u> of the dose, expressed as %.

In the "utilization" model all parameters are measured or calculated by means of a balance trial, which lasts for several days. The daily flows are obtained by averaging over the trial period. In the "isotope" model a dis-

tinct dose is followed over a certain period of time. The dose is distributed over three main compartments: body, faeces and urine. The gastrointestinal tract can be seen as part of the body compartment, but also as the barrier between the "world outside" (lumen) and the body compartment. Generally the amount of TE taken up in the body including the gastrointestinal tract is defined as "uptake" and the amount of TE taken up in the body excluding the gastrointestinal tract as "transfer" or "absorption", thus taking both views into account.

A serious problem in the "isotope" model is the time-dependence of isotope absorption, retention and excretion. Because each investigator has its own idea about the optimum time for determining these parameters, a large range of experimental approaches can be found in literature, each having its own but limited value One approach makes use of a term "apparent absorption", which is derived from the apparent absorption D from the "utilization" model and which is defined as the difference between administered activity and faecal excretion during a period long enough for excretion of unabsorbed activity from the gastrointestinal tract to occur [8] Again, the disadvantage of such an approach is the time dependence of the measured value, choice of the period of measurement.

A related problem in the "isotope" model is that the process of absorption and faecal endogenous excretion of the isotope may overlap in time, so it is impossible to distinguish between the unabsorbed isotope and the absorbed isotope excreted into the faeces. This problem can be overcome by determining endogenous excretion after parenteral administration of the isotope.

3.4. The time-independent "isotope" method of Heth and Hoekstra (H&H).

Heth and Hoekstra [9] have developed a time-independent isotope method for measuring TE absorption The method is based on a comparison between the kinetic behaviour of orally and parenterally administered isotope The basic assumption is that the fraction of the TE dose that is absorbed (A) behaves kinetically identical to a parenterally administered TE dose. This holds true when the speed of entrance of TE from the gut into the circulation is comparable to that of injected TE from the site of injection and when after absorption the two TE doses are in a comparable chemical form and their amounts not too different.

The kinetic behaviour of orally and parenterally administered TE is described by the retention curves in which the retention, defined as the amount of radioactive TE in the living animal, expressed as % of the dose and measured by whole-body counting, is plotted on a semilogarithmic scale against time (Fig. 5)

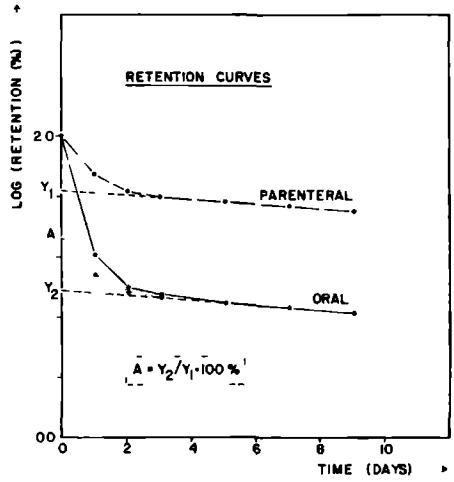


Fig. 5 Determination of the absorption A according to the method of Heth and Hoekstra [9].

It can be seen that after some time the curves become linear and parallel, which indicates that the retained TE isotope has been equilibrated with TE pools in the body. The retention decreases exponentially with time:

$$R = Y \times e^{-\lambda t} \quad (22), \qquad \text{in which:} \qquad \lambda = \frac{\ln(2)}{t_{1/2}} = \frac{0.693}{t_{1/2}} \quad (23)$$

Here $t_{1/2}$ is the physiological half-life of the TE and Y represents the fraction of the dose which is equilibrated with the body TE. For parenteral doses $Y=Y_1$ and for oral doses $Y=Y_2$. According to the basic assumption of identical behaviour of absorbed and injected TE, fraction Y is the same for an injected dose as for the absorbed part of an oral dose (A):

$$\frac{Y_1}{100\%} = \frac{Y_2}{A} \quad (24), \qquad \text{or.} \qquad A = \frac{Y_2}{Y_1} \times 100\% \quad (25)$$

In Fig 5, A is found from the retention curve after oral administration by extrapolation to the ordinate along a curved line parallel to that found for the retention curve after parenteral administration A is that part of the dose which is actually absorbed and is retained in a way comparable to the retention of the parenteral dose.

3.5. Adaptation of the "isotope" method of H&H for use in the present study.

If the absorption capacity of an organism for a TE or the bioavailability of a certain form of TE is to be investigated, then both Y_1 and Y_2 should be measured in order to calculate A according to equation (25). Y_1 and Y_2 may, however, have value in their own right as parameters for retention and absorption, respectively. Although the absorption A represents the true absorption of TE, it yields no information about the amount of TE isotope finally taken up in and equilibrated with the TE body pools. Furthermore, A can only be calculated from Y_1 and Y_2 under restricted conditions, i.e., those that assure identical kinetic behaviour of absorbed and injected TE (see Section 3.4).

Because of unfamiliarity with processes of TE absorption and metabolism the appreciation of such restrictions may be speculative, limiting the value of A. It was therefore considered useful to introduce the intercepts Y_1 and Y_2 as metabolic parameters. They are time-independent measures of the fraction of injected or absorbed TE, that has been equilibrated with TE pools in the body. Y_2 and Y_1 are not equal to the true absorption or retention. As concerning the TE tracer, however, they represent the ultimate result of the absorption or retention processes and may reflect changes in these processes by external and internal conditions. Y_1 and Y_2 can be measured independently of each other, except when the values are also used to calculate A.

For convenience the quantities Y_2 and Y_1 were given names, viz., apparent absorption (A_a) and apparent retention (R_a), resp., as they are not equal to absorption and retention, but reflect the process of absorption and retention. Furthermore, the absorption A was called true absorption (A_t) to distinguish it from the apparent absorption A_a. Equation (25) can now be rewritten as:

$$A_t = \frac{A_a}{R_a} \times 100\% \qquad (25a)$$

Fig. 6 shows how the new parameters can be calculated from the retention curves. Again, A_t should only be calculated, using equation (25a), when A_a and R_a are obtained with comparable quantities of absorbed and injected TE, respectively. When conditions of administration are not too complex, it is possible to correct A_t for differences between the amounts of absorbed or injected TE, when the dose or concentration dependence of R_a is known (see Section 4.5). This correction should be used with prudence.

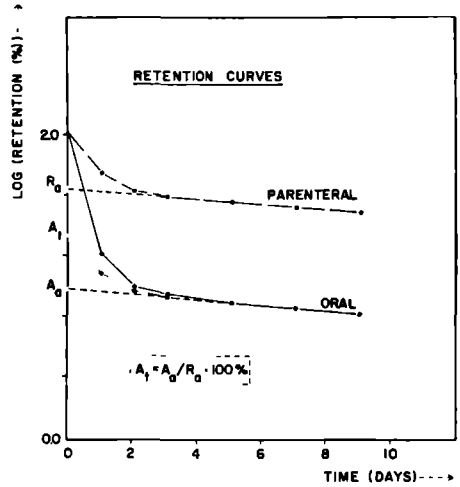


Fig. 6 Determination of the apparent absorption A_a, apparent retention R_a and true absorption A_t (present study).

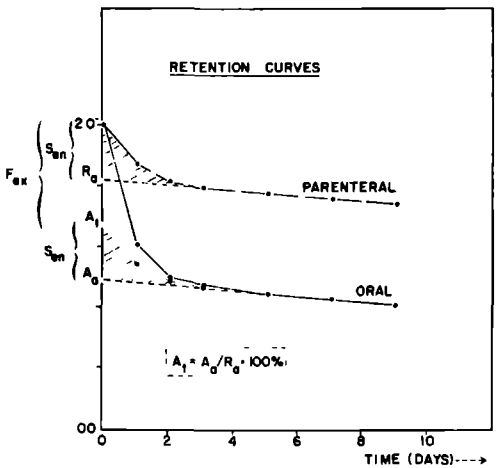


Fig. 7 Corresponding areas under the retention curves of orally and parenterally administered trace elements.

For a good understanding of the physiological meaning of A_a and R_a, an additional explanation of the structure of the retention curves may be helpful. A parenterally administered dose of tracered TE can be divided into two fractions: a rapidly excreted non-equilibrated fraction (defined as S_{en} ; "en" standing for "endogenous") and a slowly excreted fraction, equilibrated with TE pools in the body (R_a) (Fig. 7). The slow excretion of R_a is described by equation (22a):

$$R = R_a \times e^{-\lambda t} \qquad (22a)$$

An orally administered dose of tracered TE can be divided into three fractions: the 2nd and 3rd identical to those described above and together representing the truly absorbed fraction of the dose (A_t), the 1st being the rapidly excreted non-absorbed fraction of the dose (defined as F_{ex}; "ex" standing for "exogenous"). The two fractions of the truly absorbed part of the dose (A_t) represent the rapidly excreted non-equilibrated fraction (S_{en}) and the slowly excreted equilibrated fraction (A_a) (Fig. 7). The slow excretion of A_a is described by equation (22b):

$$R = A_a \times e^{-\lambda t} \qquad (22b)$$

If retention curves should be measured over a much longer period than about 10 days, as in Fig. 5-7, in addition one or more components with even longer physiological half-lifes could be found, representing more stable pools of body TE. These components could also be described with equation (22b). A_a and λ would become smaller for each additional component revealed.

Balance studies ("utilization" model) are time-consuming and very sensitive to TE contamination. Experiments with tracers ("isotope" model), on the other hand, are more sensitive to changes in experimental conditions than balance studies. This is partly because in balance studies homeostatic regulation mechanisms act to compensate for changes in uptake or excretion, partly because in such studies small differences between intake and excretion are difficult to measure. Such small differences could be extremely important on the long run. Therefore, for the detection of factors that may influence TE absorption and retention (e.g., Ca and Mg) the "isotope" method was preferred. The parameters apparent absorption (A_a), apparent retention (R_a) and (to some extent) true absorption (A_t) were used in the metabolic experiments as parameters indicative to absorption and retention of TE's and sensitive to influences of introduced variables.

3.6. Analogues between the "utilization" model of W&K and the "isotope" method used in the present study.

Apart from the essential difference between flows (mass/unit of time) and fractions of the dose (%) the "utilization" model of W&K and the "isotope" method used in the present study show some interesting analogies. These are listed in Table 2 and Table 3.

Table 2 Analogous parameters between the "utilization" model of W&K and the "isotope" method used in the present study.

"Utilization" model (W&K)		"Isotope" method (present study)	
Symbol (m/t) [2]	Term	Symbol [1] (%) [3]	Term
A	Actual absorption	A_t	True absorption
R	Apparent retention	R (t)	Retention
		R_a	Apparent retention
		A_a	Apparent absorption
F	Faecal excretion	F (t)	Faecal excretion
F_e	Endogenous faec. excr.	$F_{en}(t)$	Endogenous faec excr.
F_i	Exogenous faec. excr.	F_{ex}	Exogenous faec. excr.
U	Urinary excretion	U (t)	Urinary excretion
V	Various losses	V (t)	Various losses

[1] X (t) means X is function of time [2] (m/t) = (mass/unit of time)
[3] (%) = fraction of the dose.

Table 3 Analogous relationships between the "utilization" model of W&K and the "isotope" method used in the present study.

"Utilization" model (W&K) Relationship	"Isotope" method (present study) Relationship
$F = F_i + F_e$	$F = F_{ex} + F_{en}$
$A = I - F_i$	$A_t = 100\% - F_{ex}$
$R = I - F - U - V$	$R = 100\% - F - U - V$
$R = I - F_i - S_e$	$\begin{cases} R_a = 100\% - S_{en} \text{ and } F_{ex}=0 \\ A_a = 100\% - F_{ex} - S_{en} \end{cases}$

It should be noted, that the apparent absorption A_a is not analogous to the apparent absorption of the "utilization" model (Section 3.1) or of the time-dependent "isotope" model (Section 3.3). An analogy exists, however, when U and V are negligible. This is the case for some TE's (Zn, Cu, Cd), but not for others (Se, Co, Mn, Pb).

Although in theory A and A_t are analogous parameters, Evans et al. [10] have demonstrated, that A and A_t may differ significantly. The reason for this difference is that when the absorption of an isotope is tested by oral administration, the isotope may be diluted in the intestinal lumen by endogenous excretion of the TE concerned. Such a dilution may reduce the absorption of the isotope, when this absorption is concentration dependent. This subject is discussed more extensively in Section 4.3 and 4.5 and Appendix II.

Literature cited.

1. Weigand, E. & Kirchgessner, M. (1977) Analysis of a dynamic factorial model on trace element utilization. Z. Tierphysiol., Tierernährg. u. Futtermittelkde 39, 325-341.
2. Weigand, E. & Kirchgessner, M. (1980) Total true efficiency of zinc utilization: determination and homeostatic dependence upon the zinc supply status in young rats. J. Nutr. 110, 469-480.
3. Weigand, E. & Kirchgessner, M. (1976) Radioisotope dilution technique for determination of zinc absorption in vivo. Nutr. Metab. 20, 307-313.
4. Weigand, E. & Kirchgessner, M. (1976) ^{65}Zn-labeled tissue zinc for determination of endogenous fecal zinc excretion in growing rats. Nutr. Metab. 20, 314-320.
5. Forbes, R.M. & Parker, H.M. (1977) Biological availability of zinc in and as influenced by whole fat soy flour in rat diets. Nutr Rep. Int. 15, 681-688.
6. Momcilovic, B., Belonje, B., Giroux, A. & Shah, B.G. (1975) Total femur zinc as a parameter of choice for a zinc bioassay in rats. Nutr. Rep. Int. 12, 197-203.
7. Franz, K.B., Kennedy, B.M. & Fellers, D.A. (1980) Relative bioavailability of zinc using weight gain of rats. J. Nutr. 110, 2263-2271.
8. Aamodt, R.L., Rumble, W.F. & Henkin, R.I. (1983) Zinc absorption in humans. Effects of age, sex, and food. In: Nutritional bioavailability of zinc, pp. 61-82, G.E. Inglett, ed., American Chemical Society, Washington D.C., USA.
9. Heth, D.A. & Hoekstra, W.G. (1965) Zinc-65 absorption and turnover in rats. I. A procedure to determine zinc-65 absorption and the antagonistic effect of calcium in a practical diet. J. Nutr. 85, 367-374.
10. Evans, G.W., Johnson, E.C. & Johnson, P.E. (1979) Zinc absorption in the rat determined by radioisotope dilution. J. Nutr. 109, 1258-1264.

CHAPTER 4

ROLE OF EXPERIMENTAL CONDITIONS IN TRACE ELEMENT METABOLIC STUDIES

This Chapter contains a number of studies on fundamental aspects of trace element metabolic research, particularly on some experimental conditions that may influence the results of metabolic experiments.

4.1. Introduction.

During the last decade investigation of the metabolism of trace elements (abbr. TE's), including factors that influence it, by means of (radioactive or stable) isotopes of the TE's has proven to be very successful. Tracers allow the studying of TE metabolic pathways and may give detailed information of the metabolic fate of defined quantities of TE's. Laboratory animal studies using radiotracers have without doubt surpassed conventional balance trials in number, although the latter still can give essential additional information. Compared to balance studies, however, tracer studies provide a rapid and sensitive method to detect factors that may influence TE absorption and retention.

The consequence of the sensitivity of radiotracer studies is that the experimental conditions in such studies should be standardized rigorously. The results of radiotracer studies are often difficult to intercompare because of differences in such experimental conditions. These differences can be inherent to the subject of experiment (e.g., its TE status) or to the experimental approach (e.g., the mode of TE administration, fasting conditions, composition of TE dose, etcetera).

The TE status of mice depends strongly on the type of diet fed. The mineral and TE contents of commercially available stock diets for laboratory rats and mice are generally in excess of requirements. In TE studies such high mineral and TE levels in the food are undesirable. Therefore, a purified diet should be used rather than a stock diet. In Section 4.2 the requirements are formulated for a purified diet to be used in the study of interactions between minerals and TE's. The composition is described of a new diet that meets these requirements and that has been used throughout the present study (RIVM-CB purified diet; CB = casein basis).

At the moment of receipt from the breeding institute mice are adjusted to the stock diet on which they have been raised and maintained. Prior to experimentation they should be adequately adapted to the purified diet to avoid an influence of the preceding stock diet on the experiments to be done. The current literature offers no information on how to choose an adequate adaptation period. In Section 4.3 a study is described on the influence of a purified diet on TE intake and absorption and especially on the body content

of Zn in mice. Also the adaptation time needed for stabilization of ^{65}Zn absorption and retention is discussed.

For most TE's food is the main source of dietary intake. In fundamental research on TE absorption, administration of TE's in food is not always convenient, because food introduces many variables that may influence the absorption. To avoid this an aqueous solution of the TE can be intubated into the empty stomach of fasted mice. In the study reported in Section 4.4 this mode of administration is compared with TE administration in food and in drinking water. In view of the possible influence of food on TE absorption also the influence of fasting conditions before and after TE administration is discussed. In the same report four modes of parenteral administration are compared to allow the choice of a both representative and convenient way for determination of the apparent retention of TE's.

In the last 10 years many tracer studies reported data on the absorption and retention of TE's. Little knowledge is available, however, concerning the relation between absorption and retention of the tracer and the concentration of the TE in the administered dose. In the study described in Section 4.5 the behaviour of Zn, Cu, Se and Pb is discussed with regard to this aspect.

Section 4.6 reports a detailed study on the intestinal passage and absorption of Zn and Cu in mice after simultaneous intubation into the stomach. In this study also a comparison is made with some experiments with rats to show the animal specificity of the results.

The two most current methods of parenteral Zn isotope administration, viz., intravenous and intraperitoneal injection, are compared in Section 4.7 with respect to the tissue distribution of Zn at two different concentrations of Zn in the dose.

4.2. PAPER I

A NEW DIET FOR TRACE ELEMENT RESEARCH WITH RATS AND MICE

A.A. Van Barneveld and H. Morse

ABSTRACT A purified diet for rats and mice, developed for trace element research, is presented. Possibilities for special applications are indicated.

Introduction

Commercially available "natural ingredient" diets for laboratory rats and mice contain traditionally large and variable amounts of minerals and trace elements. They should not be used in trace element research, because:
- animals may become insensitive to small metabolic effects by overloading with minerals and trace elements from the diet;
- interfering interactions may occur between trace elements mutually and between trace elements and minerals;
- naturally occurring complexing agents in the raw food components (such as fibrous matter) may influence mineral and trace element metabolism.

Requirements

For trace element studies involving laboratory animals there is a need for a diet which meets the following requirements:
- it should be adequate for maintenance, growth and breeding;
- it should have a constant composition, based on single (large) batches of commercially available refined ingredients;
- it should contain minimum adequate amounts of minerals and trace elements;
- it should contain a range of trace elements as complete as possible;
- it should also permit the possibility of preparation with deficient or extremely low levels of one or more trace elements;
- it should not contain large amounts of amino acids or specific complexing agents (like phytate from soya, cereal or other plant sources).

IRI-CB purified diet

Based upon these requirements a fully adequate diets for rats and mice (IRI-CB purified diet) was formulated, composed of refined, relatively pure, commercially available ingredients (Table 1). The presence of protein and polysaccharide in combination with non-digestible fiber (α-cellulose) promotes a normal gastrointestinal function. The mineral content is fairly low but adequate.

IRI-OBL purified diet

A Cu/Zn-low variant of the IRI-CB diet (IRI-OBL purified diet) was formulated by replacing casein by ovalbumin (which contains a lower level of trace elements) and by omitting Cu and Zn from the trace element premix. The resulting concentration of Cu and Zn in the diet are 0.9 µg/g Cu and 1.5 µg/g Zn, low enough to produce Cu- and Zn-deficiency. An increased effect can be achieved by adding Zn as a Cu-antagonist (for Cu-deficiency) or phytic acid as a not absorbable Zn-complexing agent (for Zn-deficiency). The IRI-OBL diet can be used whenever low Cu- or Zn-values are required.

When ovalbumin is used there is no need for methionine supplementation. Ovalbumin should be heated before use at 120° for 4 hours to inactivate avidin, a protein that irreversibly binds the vitamin biotin. The biotin-inactivating capacity is then reduced from 3 to 0.4 µg biotin/g ovalbumin.

Netem-80 trace element premix

An extensive trace element premix (Netem-80: Netherlands trace element mixture) was incorporated in both diets with the necessary modifications for the low Cu/Zn-variant (Table 2). Analytical data of the complete diets are shown in Table 3. The trace element contents of the diets are not excessive. When rats are used the Zn content should be raised to 50 µg/g to maintain the integrity of the germinal epithelium in males.

Growth and breeding results

Growth curves (Fig. 1) and breeding characteristics (Table 4) of Swiss Random mice fed the IRI-CB diet are satisfying.

Comparison with the AIN-76 diet

The diets formulated deviate from the recommendations *) of the AIN (American Institute of Nutrition) in the following:

- the trace element salt premix is extended with a series of less commonly used but highly significant trace elements (Table 3): V, Mo, Ni, Sn, Co, As, B, F;
- sodium meta-silicate is added;
- the vitamin premix is adjusted (Table 5);
- sucrose is replaced by glucose to improve pelletization of the diet.

*) Journal of Nutrition 107 (1977) 1340-1348.

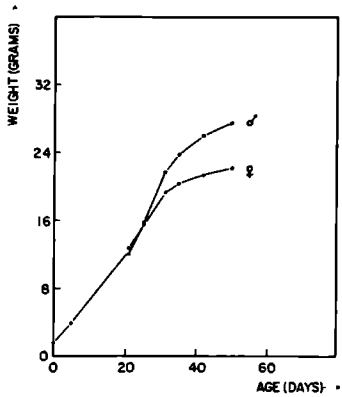


Fig. 1 Growth curves of male and female Swiss Random mice fed the IRI-CB diet (pellets).

Table 1 IRI-CB purified diet (for rats and mice). [1]

Ingredient	%	
Glucose	50.45	
Corn starch	15.0	
Casein	20.0	[2]
Sun flower seed oil	4.0	
Fiber (α-cellulose)	5.0	
dl Methionine	0.2	[2]
Choline chloride	0.3	
Mineral mix:		
Sodium diphosphate	1.5	
Potassium chloride	0 7	
Calcium carbonate	1.0	
Magnesium sulphate	0.5	
Sodium meta-silicate	0.25	
Trace element salt mix (Netem-80):	0 1	[2]
Vitamin mix in glucose	1.0	

[1] Sufficient for maintenance, growth and breeding.

[2] For preparation of the Cu/Zn-low IRI-08L purified diet casein is replaced by ovalbumin; dl methionine is omitted; Cu and Zn are omitted from the trace element salt mix.

Table 2 Netem-80 [R] trace element salt mixture. [1]

Element	µg/g (diet)	Chemical	g/kg mix
Fe	60	Fe_2O_3	85.9
Mn	50	$MnSO_4.H_2O$	153.8
Zn	20 [2]	ZnO	25.6
Cu	10	$CuCO_3.Cu(OH)_2$	17.4
Ni	2.5	$NiCl_2.6H_2O$	10.12
F	2.5	NaF	5.52
Cr	2.0	$CrCl_3.6H_2O$	10.25
Sn	2.0	$SnCl_2.2H_2O$	3.80
I	0.3	KIO_3	0.506
Mo	0.2	$Na_2MoO_4.2H_2O$	0.504
Se	0.15	Na_2SeO_3	0.329
V	0.10	NH_4VO_3	0.230
As	0.10	$Na_2HAsO_4.7H_2O$	0.416
Co	0.10	$CoSO_4.7H_2O$	0.477
B	0.10	$Na_2B_4O_7.10H_2O$	0.882
		Glucose to make 1,000 g	

[1] For preparation of the Cu/Zn-low IRI-OBL diet Cu and Zn are omitted.
[2] Recommended for rats: 50 µg/g.

Table 3 Trace element composition of the Netem-80 premix; analytical data [1] of the IRI-CB and IRI-OBL purified diets; comparison with the AIN-76 diet [2] (all concentrations are given in µg/g).

Element	Netem-80 [3]	IRI-CB (powder)	IRI-CB (pellet)	IRI-OBL [4] (powder)	AIN-76
Fe	60.	77.	84.	66.	35.
Mn	50.				54.
Zn	20. [5,6]	21.6	24.5	1.49	30.
Cu	10. [5]	9.3	10.3	0.88	6.
Ni	2.5	1.8	2.3	2.4	-
F	2.5				-
Cr	2.0	1.4	2.1	1.8	2.0
Sn	2.0				-
I	0.3				0.2
Mo	0.2	0.16	0.24	0.20	-
Se	0.15	0.17	0.22	0.36	0.1
V	0.1				-
As	0.1	0.13	0.13	0.13	-
Co	0.1	0.08	0.12	0.12	-
B	0.1				-

[1] Neutron activation analysis: Dr.ir. P.S. Tjioe, Interuniversity Reactor Institute, Delft. [2] Recommendations of the American Institute of Nutrition. [3] Resulting concentrations in the diet when the premix constitutes 0.1% of the diet. [4] Low Cu/Zn-variant of the IRI-CB diet. [5] For preparation of the IRI-OBL diet Cu and Zn are omitted. [6] Recommended for rats: 50 µg/g.

Table 4 Breeding characteristics of Swiss Random mice fed the IRI-CB diet (pellets) starting 2 weeks before breeding.

	1st Generation 1st Litter	2nd Litter	2nd Generation 1st Litter
No. of females	10	10	10
No. of litters	10	10	10
No. of pups/litter	7.9 ± 2.2	10.0 ± 3.5	8.1 ± 2.8
Ratio male/female	0.88	0.87	
% Survival	95%	94%	99%
Weight on 21st day: male	12.0 ± 2.5	11.6 ± 1.8	
female	12.7 ± 1.6	10.8 ± 1.6	

Table 5 Vitamin premix. [1]

1% of vitamin premix provides the following concentrations per kg diet:

Vitamin A (retinyl acetate)	16,000 IU
Vitamin D_3 (cholecalciferol)	1,400 IU
Vitamin K_3 (menadione)	12 mg
Vitamin K_1 (phylloquinone)	2 mg
Vitamin E (dl α-tocopheryl acetate)	85 mg
Thiamine HCl	20 mg
Riboflavine	12 mg
Pyridoxine HCl	15 mg
Niacin	40 mg
dl Calcium pantothenate	35 mg
Vitamin B_{12}	50 µg
d Biotin	400 µg
Folic acid	8 mg
Myo-inositol	500 mg

[1] Choline is added separately.

4.3. PAPER II

INFLUENCE OF APPLICATION OF A PURIFIED DIET ON ABSORPION OF ^{65}Zn, ^{64}Cu, ^{54}Mn, ^{60}Co, ^{203}Pb, ^{115m}Cd AND ^{75}Se, AND PARTICULARLY ON METABOLISM OF ZN IN MICE

A.A. Van Barneveld and C.J.A. Van den Hamer

ABSTRACT A purified diet, which was specially composed for application in trace element research with rats and mice, was tested for its influence on trace element metabolism in mice. The diet contained relatively low, but adequate levels of minerals and trace elements. Mice which were fed this diet during two weeks absorbed significantly more ^{64}Cu, ^{65}Zn, ^{60}Co, ^{203}Pb and $^{75}SeO_3^{2-}$ from an intubated solution than mice fed a stock diet. These differences could be explained partly by homeostatic adaptation to a lower trace element intake, partly by isotope dilution effects. Differences in ^{54}Mn and ^{115m}Cd absorption were not significant The organ distributions of the isotopes were affected to various degrees. The metabolism of zinc after a switch from the stock diet (containing 92 µg Zn/g) to the purified diet (containing 22 µg Zn/g) was investigated in more detail Such a switch reduced the intake of dietary Zn by 76%, which was not compensated by a higher absorption of Zn. Both in mice fed the stock diet and in mice fed the purified diet during 4 weeks the content of stable zinc in kidneys and skeletal muscle slowly decreased with age without showing an influence of the type of diet fed. Liver Zn, however, showed an additional decrease in mice fed the purified diet Under slightly different conditions adaptation of mice to the purified diet during 4 weeks caused a gradual decrease in both liver and kidney Zn. During this adaptation period the apparent absorption of ^{65}Zn was relatively constant with exception of a lower value after 1 day of adaptation, probably due to isotope dilution. No relation between apparent ^{65}Zn absorption and body Zn could be demonstrated. The apparent retention increased slightly after one week An adaptation period of 2 weeks was considered adequate for investigation of ^{65}Zn metabolism.

Introduction

Stock diets may contain high and variable levels of minerals and trace elements (TE's) [1,2] and TE complexing agents [3], which may interfere with TE absorption [4]. Therefore, the use of a purified diet instead of a stock diet in animal studies on TE metabolism is generally accepted Such a diet offers the advantage of a constant, well-defined and reproducible composition and the possibility of lowering or omitting diet components of interest

Several studies have shown an influence of the type of diet on the

results of metabolic TE experiments in animals. Oh and Whanger [5] have found that rats which were fed a purified diet for 3 weeks absorbed more ^{65}Zn from an oral dose than rats which were fed a stock diet during the same period. Mylrois et al. [6] have shown that rats consuming a purified diet were more susceptible to toxic effects of lead in the drinking water than rats consuming a stock diet. This was probably due to an increased absorption of lead. In absorption studies with lead, cadmium and strontium in rats [7-9] it has been shown that absorption of these metals from an oral dose was higher in rats fed human foods than in rats fed stock diet.

In an earlier report [10] we formulated the requirements of a purified diet to be used in the study of interactions between minerals and TE; as a result a purified diet was composed, characterized by a low but adequate content of minerals and TE's (IRI-CB purified diet; CB=casein basis). The influence of this diet on the absorption of 7 TE's was investigated in mice with the aid of radiotracers. The metabolism of zinc after a switch from a stock diet to the purified diet was investigated in more detail. The choice of an adequate adaptation time after introduction of a purified diet is discussed.

Materials and methods

Diets. Two types of diet were used: an unrefined commercial stock diet (SRMA) and a purified diet (IRI-CB). Both diets were obtained from Hope Farms BV, Woerden, The Netherlands. The composition of the IRI-CB diet is given in Table 1. Table 2 shows the mineral and TE contents of both diets.

Materials. Pepsine (35,000 U/g) was obtained from Merck, Darmstadt, BRD. Pancreatine (Grade VI from porcine pancreas) and taurocholic acid (sodium salt; approx. 98%) were obtained from Sigma, St. Louis, USA.

Isotopes. Carrier-free ^{65}Zn was obtained from The Radiochemical Centre, Amersham, UK. $^{75}SeO_3^{2-}$ (159 Ci/g Se), ^{115m}Cd (0.2 Ci/g), ^{60}Co (77.5 Ci/g) and carrier-free ^{54}Mn and ^{203}Pb were obtained from New England Nuclear, Boston, Mass., USA. ^{64}Cu (1 Ci/g) was prepared by neutron activation of Cu (purity: 99.99%) in the Interuniversity Reactor Institute, Delft, The Netherlands.

Table 1. Composition of IRI-CB purified diet

Ingredient	%
Glucose	60.45
Corn starch	15.0
Casein	20.0
Sun flower seed oil	4.0
Fiber (α-cellulose)	5.0
dl Methionine	0.2
Choline chloride	0.3
Minerals [a]	3.95
Trace element mix (NETEM-80) [c]	0.1
Vitamin mix [b]	1.0

[a] Minerals (%) : $NaH_2PO_4.2H_2O$, 1.5; KCl, 0.7; $CaCO_3$, 1.0; $MgSO_4.7H_2O$, 0.5; $Na_2SiO_3.9H_2O$, 0.25. [c] Composition in mg/kg diet : Fe_2O_3, 85.9; $MnSO_4.H_2O$, 153.8; ZnO, 25.6; $CuCO_3.Cu(OH)_2$, 17.4; $NiCl_2.6H_2O$, 10.12; NaF, 5.52; $CrCl_3.6H_2O$, 10.25; $SnCl_2.2H_2O$, 3.80; KIO_3, 0.506; $Na_2MoO_4.2H_2O$, 0.504; Na_2SeO_3, 0.329; NH_4VO_3, 0.230; $Na_2HAsO_4.7H_2O$, 0.477; $CoSO_4.7H_2O$, 0.416; $Na_2B_4O_7.10H_2O$, 0.882; glucose to make 1000 mg/kg diet. [b] Composition in IU or mg/kg diet : Retinyl acetate, 16,000 IU; cholecalciferol, 1,400 IU, Menadione, 12; phylloquinone, 2; dl α-tocopheryl acetate, 85; thiamine HCl, 20; riboflavine, 12; pyridoxine HCl, 15; nicotinamide, 40; dl calcium pantothenate, 35; vitamin B 12, 0.05; d biotin, 0.4; folic acid, 8; myo-inositol, 500 mg.

Animals. Female Swiss Random mice were obtained from the Central Institute for the Breeding of Laboratory Animals-TNO, Austerlitz, The Netherlands, at the age of 4 weeks, unless mentioned otherwise. These mice had been raised on SRMA stock diet. They were housed in macrolon cages covered with stainless steel lids and provided with glass bottles with stainless steel drinking nipples. Food and tap water were given ad libitum.

Table 2 Mineral and trace element composition of SRMA unrefined stock diet and IRI-CB purified diet. Data are compared with the recommendations of the American Institute of Nutrition (AIN-76 [11,12]).

Element	SRMA [1]	IRI-CB [2]	AIN-76
		µg/g	
Na	2200	2610	1020
K	7400	3670	3600
Ca	9300	4000	5200
Mg	2300	490	500
Si	- [3]	250	- [4]
P	7100	2980	4000
Fe	196	60 (84) [1]	35
Mn	70	50	54
Zn	92	20 (24) [1]	30
Cu	25	10 (10) [1]	6
Ni	1.1	2.5	- [4]
F	- [3]	2.5	- [4]
Cr	1.6	2.0	2.0
Sn	- [3]	2.0	- [4]
I	0.35	0.3	0.2
Mo	1.5	0.2	- [4]
Se	0.5	0.15 (0.2) [1]	0.1
V	- [3]	0.1	- [4]
As	0.8	0.1	- [4]
Co	0.3	0.1 (0.1) [1]	- [4]
B	- [3]	0.1	- [4]
Cd	0.17	- [4] (0.02) [1]	- [4]
Pb	- [3]	- [4]	- [4]

[1] Obtained by analysis after pelletization [2] Added as salt mixture to the basic components [3] Not determined [4] Not included

Measurements. Trace element concentrations in the diets were measured by neutron activation analysis according to Tjioe et al. [13]. Stable zinc concentrations in liver, kidneys, muscle and digested food samples were analyzed by atomic absorption spectrometry after chemical destruction with ultrapure nitric acid and hydrogen peroxide.

Whole-body retention of isotopes was measured in a whole-body counter (a combination of two 3"x3" NaI crystals with 1.5"x2" wells, positioned oppositely, thus forming the cavity in which the mouse could be placed). Signals from both crystals were summed. Whole-body retention (R) was measured on day 0, 1, 2, 3, 5, 7, 9 (R_0 = 100%), except for ^{64}Cu which, because of its physical half-life of 12.8 hours, could only be measured until day 3. Mice which, on basis of their isotope retention on day 9 (R_9), were recognized as "outlier" according to Chauvenet's criterion [14] were not further included in the calculations.

The mean retention per group of animals was plotted on a semi-logarithmic scale against time. Apparent absorption (A_a) of orally administered isotopes and apparent retention (R_a) of parenterally administered isotopes were determined from the retention curves acccording to the method of Heth and Hoekstra [15] (Fig. 1). For ^{64}Cu no complete retention curve could be measured, so the retention on day 3 (R_3) was used as an approximation of A_a. After 9 days the mice were sacrificed and 5 organs (liver, kidneys, spleen, pancreas and heart) were collected and their isotope content measured in an auto-gamma scintillation spectrometer (Packard 5120).

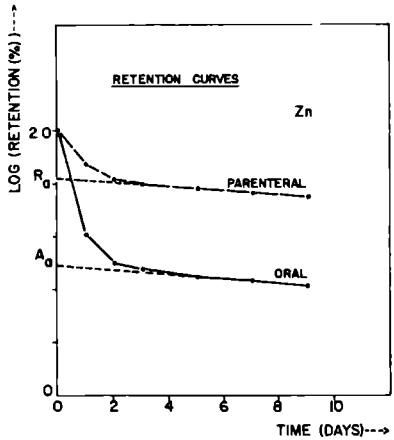


Fig. 1 Determination of apparent absorption (A_a) and apparent retention (R_a) from the retention curves, according to the method of Heth and Hoekstra [15]. Typical retention curves of ^{65}Zn are shown as an example.

All measurements of radioactivity were corrected for radioactive decay and for differences in counting geometry by comparison with standard solutions of the isotopes

Experiment 1. To investigate the influence of the purified diet on the absorption of seven trace elements 7 groups of 10 mice were fed the stock diet on which they had been raised, and another 7 groups of 10 mice were fed the purified diet (pelleted), both ad libitum during 2 weeks. After 20 hours of fasting each 10 mice of both dietary groups received by intragastric intubation one of the following 7 radioisotopes. ^{65}Zn, ^{64}Cu, ^{54}Mn, ^{60}Co, ^{115m}Cd, ^{203}Pb or $^{75}SeO_3^{2-}$ (1 µCi, 0.3 µg of the element in 0.3 ml demineralized water, except ^{64}Cu: 3 µCi, 3 µg in 0.3 ml, and ^{115m}Cd: 1 µCi, 5 µg in 0.3 ml). Isotope administration was immediately followed by whole-body counting. Six hours after isotope administration the purified diet was given to all groups and this was continued until the end of the experiment. Whole-body counting of the mice was carried out during a period of 9 days following on the day of administration. On the last day the mice were sacrificed and the organs were collected for counting of the retained isotopes

Experiment 2. A. To determine the zinc uptake from the stock diet and from the purified diet 2 groups of 10 mice, raised on the stock diet, were fed the purified diet during 5 days to clean up the gastrointestinal tract from unrefined food components. Thereupon they were fastened for 20 hours. One group was given a portion of 5 g stock diet (crumbled), the other group was given a portion of 10 g purified diet (powder). To the portions 2 µCi ^{65}Zn/g food was added (carrier-free) by thoroughly mixing with a ^{65}Zn solution (0.2 ml/g food). After 1.5 hour the portions had been consumed completely. The individual doses were measured immediately after consumption by whole-body counting and showed a standard deviation of 25% for the stock diet and 12% for the purified diet. Six hours after counting the purified diet was given ad libitum and this was continued until the end of the experiment. Whole-body counting of the mice was carried out during a period of 9 days following on the day of administration. In the individual mice no relation was observed between the absorption of ^{65}Zn and the quantity of food consumed.

B. To determine the exchangeability and bioavailability of stable Zn and ^{65}Zn in the stock diet and purified diet under conditions existing in the gastrointestinal tract 0.8 g of each type of food was, after addition of ^{65}Zn as described above, submitted to an in vitro procedure simulating peptic and pancreatic digestion according to the method of Schwartz et al [16]. The food was suspended in water, acidified to pH 1.8 by addition of HCl, and pepsine was added to a final concentration of 1%. The mixture was shaken for 1 hour at 37 °. After this peptic digestion step the suspension was adjusted to pH 6.8 by addition of sodium bicarbonate, pancreatine and taurocholic acid were added to a final concentration of 1% and 0.1%, resp., and this mixture

was shaken for another 20 minutes at 37 °. Before and after both digestion steps aliquots of the mixture were taken and centrifuged at 3000 g for 10 minutes. ^{65}Zn and total Zn were analyzed in solid residue and supernatant.

Experiment 3. To investigate the influence of the purified diet on the tissue zinc content of mice 10 mice of age 3 weeks and 10 mice of age 4 weeks were fastened for 20 hours and then sacrificed; liver, kidneys and skeletal muscle were collected. Another group of 90 mice, 4 weeks old, was divided into two groups of 45 mice; one group was fed the stock diet ad libitum, the other the purified diet (pelleted) ad libitum. At the age of 5, 6, 7 and 8 weeks 10 mice and at the age of 9 weeks the remaining 5 mice of each group were sacrificed after 20 hours of fasting; again liver, kidneys and skeletal muscle were collected. In the collected tissues zinc was analyzed by atomic absorption spectrometry.

Experiment 4. To investigate the relation between purified diet adaptation, tissue zinc content and ^{65}Zn metabolism 5 groups of 25 mice, raised on the stock diet, were fed the purified diet (pelleted) during varying periods of time, ranging from 1 to 28 days. These periods all ended on the same day; adequate periods of stock diet feeding were applied in advance (Fig. 2). This way an age effect on the outcome of the experiment was avoided. One day of adaptation was chosen as the minimum adaptation time, instead of no adaptation at all, to assure a complete removal of stock diet components from the gastrointestinal tract before administration of ^{65}Zn. At the end of the adaptation period the animals were fastened for 20 hours. Thereupon each group was divided into three sub-groups: 5 animals were sacrificed and their livers and kidneys collected for analysis of the zinc content; 10 animals received 1 µCi ^{65}Zn by intragastric intubation (0.3 µg Zn in 0.3 ml demineralized

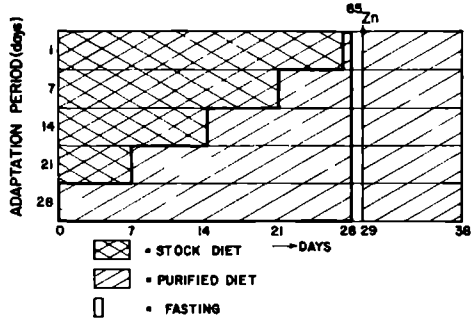


Fig. 2 Feeding scheme for the mice in experiment 3. The diet adaptation periods were started at consecutive moments so as to end on the same day and therefore at the same age of the mice.

water), another 10 animals received 0.3 µCi ^{65}Zn by intraperitoneal injection (0.3 µg Zn in 0.3 ml acetate buffer 0.05 M, pH 5.6, 0.7% NaCl). ^{65}Zn administration was immediately followed by whole-body counting. Six hours after ^{65}Zn administration the purified diet was given ad libitum and this was continued until the end of the experiment. Whole-body counting of the mice was carried out during a period of 9 days following on the day of administration.

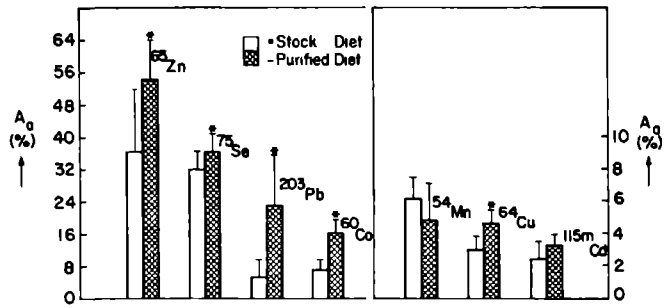


Fig. 3 Apparent absorption (A_a) of ^{65}Zn, ^{75}Se, ^{203}Pb, ^{60}Co, ^{54}Mn, ^{64}Cu and ^{115m}Cd from an intubated solution (exp. 1). The mice were fed the stock diet (blank area) or the purified diet (shaded area) during 2 weeks prior to isotope administration. *) Significantly different by Student's t-test, $P < 0.05$.

Results

Experiment 1. As shown in Fig. 3 the apparent absorption of Zn, Cu, Co, Pb and Se (as selenite), intubated into the stomach as solutions labeled with the respective radionuclides, was higher in mice fed the purified diet than in mice fed the stock diet prior to isotope administration, the absorption of Mn and Cd was not significantly influenced. The type of diet used also influenced the organ distribution of the isotopes (Table 3). For some specific isotope/organ combinations highly significant differences were found. The excess of ^{60}Co absorbed by mice fed the purified diet (Fig. 3) was mainly recovered in the liver (Fig. 4), which suggests that this organ has a storage function for inorganic Co.

Experiment 2. Table 4 shows that the apparent absorption A_a of ^{65}Zn from the purified diet was 15.4% and that from the stock diet 11.4%. The exchangeability of ^{65}Zn and stable zinc in both diets was found to be close to 100% (Table 5). The absolute apparent absorption A_a^a (µg Zn per g food) could therefore be calculated as the product of the apparent absorption A_a

Table 3 Organ distribution of intragastrically intubated isotopes in mice fed the stock diet or the purified diet prior to isotope administration (exp. 1). The data express the isotope content of the whole organ as % of the content in the whole body at the moment of sacrifice (day 9). [1]

Diet	n	Liver	Kidneys	Spleen	Pancreas	Heart
				%		
^{65}Zn:						
Stock	10	7.3±0.4	1.0±0.1	0.36±0.06	1.29±0.22	0.42±0.05
Purified	9	6.7±0.5	1.2±0.2 [3]	0.32±0.09	1.01±0.14 [4]	0.43±0.04
^{54}Mn:						
Stock	10	21.4±3.2	6.1±0.8	0.16±0.07	5.22±1.15	0.90±0.13
Purified	10	23.2±4.4	7.4±1.2 [3]	0.25±0.09 [3]	5.49±0.96	0.82±0.13
^{60}Co:						
Stock	10	36.2±7.2	9.9±1.3	0.37±0.12	1.60±0.61	1.74±0.48
Purified	10	43.1±5.7 [2]	5.5±1.8 [4]	0.22±0.04 [4]	0.82±0.16 [4]	0.82±0.30 [4]
^{203}Pb:						
Stock	9	2.2±0.6	6.8±1.2	-	0.33±0.19	-
Purified	10	2.3±0.5	6.4±1.3	-	0.12±0.10 [4]	-
^{75}Se:						
Stock	10	25.4±1.6	7.4±0.6	0.73±0.12	0.92±0.11	0.37±0.02
Purified	10	21.6±1.0 [4]	7.4±1.1	0.60±0.09 [3]	0.85±0.15	0.37±0.02

[1] Data are presented as mean±SD. [2,3,4] Difference significant according to Student's t-test, for $P < 0.05$, $P < 0.02$ and $P < 0.01$, resp.

Table 4 Apparent absorption A_a of ^{65}Zn (%) and absolute apparent absorption A_a^a of stable zinc (µg per g food) from the stock diet (crumbled) and the purified diet (powder) (exp. 2A). [1]

Diet	[Zn] [2]	n	A_a (^{65}Zn)	A_a^a (Zn)
	µg/g		%	µg per g food
Stock	92	9	11.4±1.4	10.5
Purified	22	10	15.4±1.6 [3]	3.4

[1] Data are presented as mean±SD. [2] Analyzed by atomic absorption spectrometry. [3] Difference significant according to Student's t-test, $P < 0.01$.

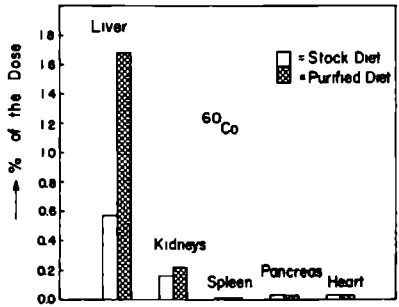


Fig. 4 Organ distribution of intragastrically intubated ^{60}Co (exp. 1). The mice were fed the stock diet (blank area) or the purified diet (shaded area) prior to ^{60}Co administration. The ^{60}Co content of the organs is expressed as % of the dose.

and the stable Zn content of the diet and was found to be 10.5 µg Zn per g stock diet and 3.4 µg Zn per g purified diet. A switch from the stock diet to the purified diet therefore reduced the zinc absorption by 68%. Due to its higher caloric value, daily ad libitum consumption of the purified diet was about 20% lower by weight than consumption of the stock diet (unpublished results), so in reality this reduction was 74%.

The difference in potential bioavailability of ^{65}Zn, shown in Table 5 (78% in the stock diet, 92% in the purified diet), was probably the result of the Zn binding capacity of undigested food components in the stock diet at pH 6.8 in the pancreatic digestion step.

Experiment 3. Fig. 5 shows that the zinc content in kidneys and skeletal muscle of mice fed the stock diet or the purified diet slowly decreased with age without showing an influence of the type of diet consumed; the zinc content in the liver showed a similar age effect, but in addition a decrease in the first week after introduction of the purified diet.

Experiment 4. Fig. 6 shows that both liver and kidney Zn in mice adapted to the purified diet decreased with increasing length of the adaptation period, suggesting a gradual depletion of Zn body stores during adaptation. The foregoing results (Fig. 5) showed a rapid decrease in liver Zn and no effect on kidney Zn after introduction of the purified diet; this discrepancy is not understood.

The apparent absorption (A_a) of ^{65}Zn from an intubated solution (Fig. 7; left) was relatively constant during adaptation; only the value after 1 day of adaptation seemed to be lower. The difference was, however, not significant. The apparent retention (R_a) of ^{65}Zn injected intraperitoneally (Fig. 7; right) increased slightly after one week of adaptation.

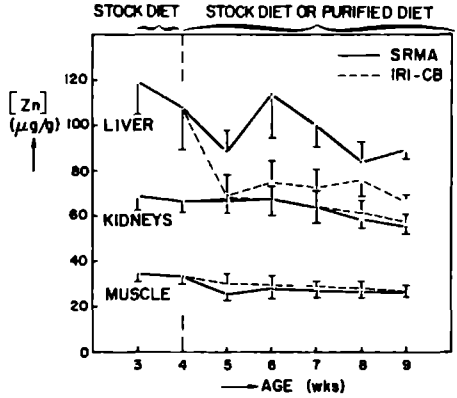


Fig. 5 Zinc content (µg/g dry weight) in liver, kidneys and skeletal muscle as influenced by age and type of diet (exp 3)

Discussion

The mineral and trace element (TE) content of the purified diet was much lower than that of the stock diet (Table 2). Among others Zn was reduced from 92 to 24 µg/g, Cu from 25 to 10 µg/g, Se from 0.5 to 0.2 µg/g and Co from 0.3 to 0.1 µg/g. The observed increase in the apparent absorption of these elements could be the result of the reduction of their intake. The apparent absorption of Mn showed no difference, probably because the Mn contents of both diets were similar (70 µg/g in the stock diet and 50 µg/g in the purified diet).

As the TE absorption was measured with the aid of radiotracers, two possible effects should be distinguished, viz., a physiological effect and an isotope dilution effect. A physiological effect could be caused by homeostatic adaptation of the absorption and excretion mechanisms to the lower TE intake from the purified diet. An isotope dilution effect could be caused by endogenous excretion of the TE in question into the intestinal lumen.

The increased apparent absorption of Se could be the result of adaptation of the Se excretion. In rats fed increasing Se levels in the diet Burk et al. [18] have found a decrease in the apparent absorption of ^{75}Se, which was caused by an elevated ^{75}Se excretion in the urine. Adaptation to dietary intake could be specific for each single trace element, but could also have an aspecific character. This will depend on the specificity of the mechanisms concerned. Co seems to share part of its absorption pathway with iron [19]. The increased apparent absorption of Co could therefore be a side-effect of

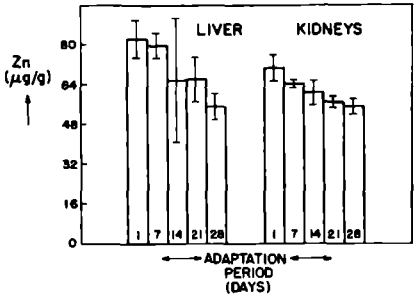


Fig. 6 Zinc content (µg/g dry weight) in liver and kidneys during adaptation to the purified diet (exp. 4). All mice had the same age at the moment of sacrifice.

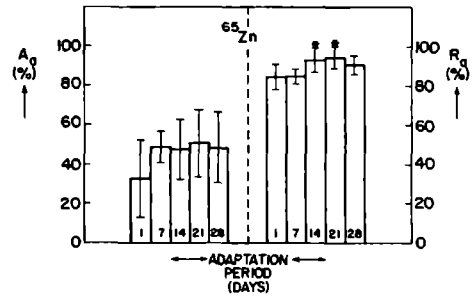


Fig. 7 Apparent absorption (A_a) and apparent retention (R_a) of ^{65}Zn during adaptation to the purified diet (exp. 4). All mice had the same age at the moment of ^{65}Zn administration. *) R_a was significantly increased with respect to R_a after 1 day of adaptation according to Student's t-test, $P < 0.05$.

adaptation of the Fe absorption to a lower Fe intake (decreasing from 196 to 77 µg/g). In the same way the increased apparent absorption of Pb could be the result of a lower Ca intake (decreasing from 9300 to 4000 µg/g) [20,21].

As mentioned earlier, radiotracer absorption can be influenced by isotope dilution in the intestinal lumen due to endogenous excretion of TE. Depending on the exchangeability between chemical forms an endogenous intraluminal TE pool may reduce the apparent absorption of the isotope. Several studies have shown that this may particularly be true for Zn [22,23]. As

Table 5 Extraction of ^{65}Zn and stable zinc from the stock diet (crumbled) and the purified diet (powder) by in vitro peptic and pancreatic digestion according to the method of Schwartz et al [16] (exp 28) The high exchangeability is in agreement with results of in vivo experiments with ^{65}Zn in intrinsically and extrinsically labeled food [17].

Diet:	Stock diet	Purified diet
Zn content (µg/g):	92	22
	%	
a. Peptic digestion.		
^{65}Zn found in residue	1.8	0.17
Zinc found in residue	2.6	<0.5
Exchangeability [1]	99 [2]	100
b. Pancreatic digestion:		
^{65}Zn found in residue	22	8
Bioavailablity	78	92

[1] All Zn in solution was assumed to be exchangeable. [2] Assuming that the 1.8% ^{65}Zn found in the bulky residue was mainly the result of inclusion of supernatant in the residue, the exchangeability was calculated to be 100% - (2.6 - 1.8)% ≅ 99%.

the stock diet contained much more Zn than the purified diet, the endogenous excretion of Zn was probably high during stock diet consumption and caused a stronger isotope dilution than during purified diet consumption. This could explain the difference in apparent absorption of ^{65}Zn. Concerning ^{64}Cu absorption, Cu is excreted with the bile, probably in a chemical form unavailable for reabsorption [24], so isotope dilution is an unlikely factor Therefore, the increase in the apparent absorption of ^{64}Cu probably results from adaptation to the lowered Cu intake. For Cd no significant difference in the apparent absorption was found. Obviously, no aspecific adaptation as for Pb occurred or was strong enough to cause an effect

The Zn content of the stock diet was four times higher than that of the purified diet, so the dietary Zn intake was strongly reduced by a switch from the former to the latter diet This difference was not compensated by an corresponding increase in the absorption of Zn (Table 4). The decreased Zn intake resulted in a reduction of the Zn content in the liver (exp 3) or in both liver and kidneys (exp. 4). This suggests that during a period of a large Zn supply (e.g., from the stock diet) these organs, particularly the liver, may contain a depot of available Zn that is depleted during a period of a smaller Zn supply (e.g., from the purified diet). The difference between the results of experiment 3 and 4 with respect to the kidney Zn content is

not understood.

In rats fed diets with various Zn levels (6-33 µg/g) during 4 weeks it was found that in the range of 12-33 µg/g the liver Zn was directly related to the Zn intake, whereas the kidney Zn was constant [25]. In another study with growing rats fed diets containing 2, 14 and 57 µg/g Zn during 8 weeks, both liver and kidney Zn were directly related to the Zn intake. The differences, however, were significant for the kidneys only [26]. Apparently the relation between the Zn content in the liver and kidneys and that in food is complex. Petering et al. [26] have suggested that some of the Zn supplied in excess of the amount needed for maximum growth, is stored in the organs as a depot and that this process is part of the homeostatic regulation of the Zn retention.

Although the Zn content in liver and kidneys continuously decreased during 4 weeks of adaptation to the purified diet, the apparent absorption of ^{65}Zn was relatively constant. Obviously there was no relation between Zn absorption and body Zn. The lower value at the first day after introduction of the purified diet was probably caused by isotope dilution by endogenous excretion of Zn still originating from the previously given stock diet. Also dilution by Zn in the mucosal cells could have occurred, because the Zn content of these cells could still be high shortly after the last stock diet feeding. The slight increase of the apparent retention of ^{65}Zn after one week of adaptation could not be attributed to a reduced isotope dilution by body Zn pools, because this would have had an opposite effect on ^{65}Zn retention. An increased affinity of the tissues for Zn is more likely. After 2 weeks of adaptation no significant changes in the apparent absorption and retention of ^{65}Zn were observed anymore.

It is concluded that in studies on the metabolism of trace elements with the aid of radiotracers the results may not only depend on the type of diet consumed prior to the experiments, but also on the time during which it is fed in advance. A certain adaptation period is desirable to avoid an influence of the preceding diet on the outcome of the experiments. The results suggest that for metabolic experiments with ^{65}Zn in mice placed on a purified diet an adaptation period of 2 weeks is sufficient for stabilization of absorption and retention of ^{65}Zn.

Acknowledgements

Prof. J.P.W. Houtman, dr. J.H.M. Van Tongeren and ir. H. Delhez are acknowledged for critical review of the manuscript. Dr. P.S. Tjioe is thanked for analyses of trace elements in diets. Mr. T. Van der Meer was very helpful in carrying out analyses of zinc by atomic absorption spectrometry.

Literature cited

1. Wise, A. (1981) The standard stock diet - fact or fiction. Nutr. Rep. Int. 23, 287-293.
2. Wise, A. & Gilburt, D.J. (1981) Variation of minerals and trace elements in laboratory animal diets. Lab. Anim. 15, 299-303.
3. Wise, A. & Gilburt, D.J. (1980) The variability of dietary fibre in laboratory animal diets and its relevance to the control of experimental conditions. Fd Cosmet. Toxicol. 18, 643-648.
4. Davies, N.T. (1979) Anti-nutrient factors affecting mineral utilization. Proc. Nutr. Soc. 38, 121-128.
5. Oh, S.-H. & Whanger, P.D. (1981) Effect of cadmium administration on absorption, retention, and excretion of zinc-65 administered to rats. Environ. Res. 26, 130-135.
6. Mylroie, A.A., Moore, L., Olyai, B. & Anderson, M. (1978) Increased susceptibility to lead toxicity in rats fed semipurified diets. Environ. Res. 15, 57-64.
7. Kostial, K. & Kello, D. (1979) Bioavailability of lead in rats fed "human" diets. Bull. Environm. Contam. Toxicol. 21, 312-314.
8. Rabar, I. & Kostial, K. (1981) Bioavailability of cadmium in rats fed various diets. Arch. Toxicol. 47, 63-66.
9. Kostial, K., Simonović, I., Rabar, I. & Landeka, M. (1980) Influence of human foods and rat diet on radiostrontium bioavailability in rats. Period. Biol. 82, 229-234.
10. Van Barneveld, A.A. & Morse, H. (1980) A new diet for trace element research. Z. Versuchstierk. 24, 24.
11. American Institute of Nutrition (1977) Report of the ad hoc committee on standards for nutritional studies. J. Nutr. 107, 1340-1348.
12. American Institute of Nutrition (1980) Second report of the ad hoc committee on standards for nutritional studies. J. Nutr. 110, 1726.
13. Tjioe, P.S., De Goeij, J.J.M. & Houtman, J.P.W. (1977) Extended automated separation techniques in destructive neutron activation analysis; application to various biological materials, including human tissues and blood. J. Radioanal. Chem. 37, 511-522.
14. De Soete, D., Gijbels, R. & Hoste, J. (1972) In: Neutron activation analysis, pp. 505-506. Chemical Analysis, Vol. 34 (Ed. Elving, P.J. & Kolthoff, J.M.). Wiley-Interscience, London.
15. Heth, D.A. & Hoekstra, W.G. (1965) Zinc-65 absorption and turnover in rats. I. A procedure to determine zinc-65 absorption and the antagonistic effect of calcium in a practical diet. J. Nutr. 85, 367-374.
16. Schwartz, R., Belko, A.Z. & Wien, E.M. (1982) An in vitro system for measuring intrinsic dietary mineral exchangeability: alternative to intrinsic isotopic labeling. J. Nutr. 112, 497-504.

17. Evans, G.W. & Johnson, P.E. (1977) Determination of zinc availability in foods by the extrinsic label technique. Am. J. Clin. Nutr. 30, 873-878.
18. Burk, R.F., Brown, D.G., Seely, R.J. & Scaief III, C.C. (1972) Influence of dietary and injected selenium on whole-body retention, route of excretion, and tissue retention of $^{75}SeO_3^{2-}$ in the rat. J. Nutr. 102, 1049-1056.
19. Becker, G., Huebers, H. & Rummel, W. (1979) Intestinal absorption of cobalt and iron: mode of interaction and subcellular distribution. Blut 38, 397-406.
20. Barton, J.C., Conrad, M.E., Harrison, L. & Nuby, S. (1978) Effect of calcium on the absorption and retention of lead. J. Lab. Clin. Med. 91, 366-376.
21. Hart, M.H. & Smith, J.L. (1981) Effect of vitamin D and low dietary calcium on lead uptake and retention in rats. J. Nutr. 111, 694-698.
22. Evans, G.W., Johnson, E.C. & Johnson, P.E. (1979) Zinc absorption in the rat determined by radioisotope dilution. J. Nutr. 109, 1258-1264.
23. Flanagan, P.R., Haist, J. & Valberg, L.S. (1983) Zinc absorption, intraluminal zinc and intestinal metallothionein levels in zinc-deficient and zinc-replete rodents. J. Nutr. 113, 962-972.
24. Solomons, N.W. (1981) Zinc and copper in human nutrition. In: Nutrition in the 1980s: Constraints on our knowledge, pp. 97-127, Alan R. Liss, Inc., New York, USA.
25. Deemung, S.B. & Weber, C.W. (1977) Evaluation of hair analysis for determination of zinc status using rats. Am. J. Clin. Nutr. 30, 2047-2052.
26. Petering, H.G., Giroux, E., Choudhury, H. & Menden, E.E. (1982) Consecutive zinc balance trials in growing rats. Biol. Tr. El. Res. 4, 221-232.

4.4. PAPER III

INFLUENCE OF ISOTOPE ADMINISTRATION MODE AND OF FOOD CONSUMPTION ON ABSORPTION AND RETENTION OF TRACE ELEMENT RADIOTRACERS IN MICE: A STUDY WITH ^{65}Zn

A.A. Van Barneveld and C.J.A. Van den Hamer

ABSTRACT The retention of ^{65}Zn was measured in mice after 3 modes of oral and 4 modes of parenteral administration. The apparent absorption of ^{65}Zn from an intubated solution, from drinking water and from food was 51%, 48% and 11%, resp. The apparent retention of parenterally injected ^{65}Zn was independent of the mode of injection (intraperitoneally, subcutaneously, intramuscularly and intravenously, resp.) (83-84%). Food consumption prior to, during, or following ^{65}Zn administration reduced the apparent absorption. Also an effect was found of the type of food consumed (purified diet or stock diet). It is concluded that in zinc absorption studies the mode of isotope administration and the conditions of food consumption must be carefully chosen and extensively described in papers. The observed effects may have implications for the estimation of human zinc uptake from the diet and for the therapeutic and diagnostic application of zinc in medicine. These conclusions may also be valid for other trace elements.

Introduction

There are several methods for investigating zinc absorption in man and animals. Much information on the zinc balance can be obtained from measurement of zinc content of food and excreta [1]. Measurement of zinc dependent biological parameters in animals (weight gain, total bone zinc and total liver zinc), as described extensively by Franz et al. [2], can be helpful in estimating relative bioavailability of zinc (RBAZ) from food components in comparison with a standard zinc source like zinc carbonate. Zinc isotopes offer the possibility of following absorption and excretion processes of a particular quantity of zinc going through a number of zinc pools in the intact organism.

In animal studies ^{65}Zn is widely used due to its convenient radiation characteristics and long half-life ($t_{1/2}$=245 days) [3-5]. In human studies not only ^{65}Zn is used [6-8], but also the shorter living ^{69m}Zn ($t_{1/2}$=13.9 hrs) [9,10]. Beside these radioactive isotopes there is a growing interest in the possibilities of the stable isotopes ^{68}Zn, ^{70}Zn and ^{64}Zn [11-15], which can be easily analyzed by neutron activation. Using these isotopes a radiation burden in the (human) subject is avoided, however, the possibility of whole-body counting is lacking and sampling techniques are required instead.

A comparative zinc absorption study in rats showed a high correlation between the results of ^{65}Zn and ^{70}Zn metabolic measurements [16].

As there is growing evidence that the Zn-intake in humans is often sub-adequate [17,18], more information is needed about the efficiency of zinc absorption from different dietary sources. The current knowledge on this subject has been reviewed recently by Solomons [18]. The recent forthcoming of short-living and stable isotopes for human studies will stimulate research activities in this field. On the other hand animal studies with ^{65}Zn will continue to provide supporting information about aspects of zinc metabolism.

In this study attention is given to the influence of the experimental conditions under which isotopes are administered on absorption data. Three modes of oral and four modes of parenteral ^{65}Zn administration are compared in mice. The effect of different conditions of food consumption on the apparent absorption and retention of ^{65}Zn are evaluated.

Methods

A purified diet (IRI-CB diet containing 22 µg Zn/g) and a stock diet (SRMA diet containing 92 µg Zn/g) were obtained from Hope Farms, Woerden, The Netherlands. The composition of the purified diet is given in Table 1. The argumentation for this composition has been published earlier [19]. Carrier-free ^{65}Zn was obtained from The Radiochemical Centre, Amersham, UK. Female Swiss Random mice, 4 weeks old, were obtained from the Central Institute for the Breeding of Laboratory Animals - TNO, Austerlitz, The Netherlands. They were housed in macrolon cages with stainless steel lids and provided the purified diet and demineralized drinking water ad libitum for two weeks, unless mentioned otherwise. After this adaptation period the mice were housed in metabolic cages and the following experiments were carried out:

Experiment 1. To test 3 different modes of oral administration 3 groups of 10 mice were fastened for 20 hours. Carrier-free ^{65}Zn was added to demineralized water, containing 1 µg Zn/ml as zinc sulphate (15.4 µM) and a trace

Table 1. Composition of IRI-CB purified diet.

Ingredient	%
Glucose	50.45
Corn starch	15.0
Casein	20.0
Sun flower seed oil	4.0
Fiber (α-cellulose)	5.0
dl Methionine	0.2
Choline chloride	0.3
Minerals [1]	3.95
Trace element mix (NETEM-80) [2]	0.1
Vitamin mix [3]	1.0

[1] Minerals (%) : $NaH_2PO_4.2H_2O$, 1.5; KCl, 0.7; $CaCO_3$, 1.0; $MgSO_4.7H_2O$, 0.5; $Na_2SiO_3.9H_2O$, 0.25. [2] Composition in mg/kg diet : Fe_2O_3, 85.9; $MnSO_4.H_2O$, 153.8; ZnO, 25.6; $CuCO_3.Cu(OH)_2$, 17.4; $NiCl_2.6H_2O$, 10.12; NaF, 5.52; $CrCl_3.6H_2O$, 10.25; $SnCl_2.2H_2O$, 3.80; KIO_3, 0.506; $Na_2MoO_4.2H_2O$, 0.504; Na_2SeO_3, 0.329; NH_4VO_3, 0.230; $Na_2HAsO_4.7H_2O$, 0.477; $CoSO_4.7H_2O$, 0.416; $Na_2B_4O_7.10H_2O$, 0.882; glucose to make 1000 mg/kg diet. [3] Composition in IU or mg/kg diet : Retinyl acetate, 16,000 IU; cholecalciferol, 1,400 IU. Menadione, 12; phylloquinone, 2; dl α-tocopheryl acetate, 85; thiamine HCl, 20; riboflavine, 12; pyridoxine HCl, 15; niacin, 40; dl calcium pantothenate, 35; vitamin B 12, 0.05; d biotin, 0.4; folic acid, 8; myo-inositol, 500 mg.

of histidine (154 µM), and administered to the fastened mice:

(1) by intragastric intubation ("i.g."; 0.3 ml/mouse);

(2) as drinking water ("d.w."; 15 ml/group);

(3) mixed with food ("f."; 2 ml + 10 g food/group).

As food the IRI-CB purified diet (22 µg Zn/g) was used.

The ^{65}Zn containing drinking water and food were made available to the groups for 1.5 hours. Immediately afterwards the consumed dose was measured by whole body counting. The dose was 1.0 µCi/mouse in group 1, and on the average 0.71 µCi and 1.68 µCi/mouse in group 2 and 3, resp. The IRI-CB diet was fed again ad libitum 6 hours after the end of the ^{65}Zn administration period until the end of the experiment.

Experiment 2. To test 4 different modes of parenteral administration 4 groups of 10 mice were fastened for 20 hours. Carrier-free ^{65}Zn was added to acetate buffer (0.05 M, pH 5.6, 0.7% NaCl), containing 1 µg Zn/ml as zinc sulphate (15.4 µM) and a trace of histidine (154 µM), and administered to the fastened mice by injection (0.3 ml/mouse):

(1) intraperitoneally ("i.p.");

(2) subcutaneously ("s.c.");

(3) intramuscularly ("i.m.");

(4) intravenously ("i.v.").

The injected dose was 0.3 µCi ^{65}Zn in all mice. The IRI-CB diet was fed again ad libitum 6 hours after injection until the end of the experiment.

Experiment 3 (see also Fig. 3). To test the influence of food consumption on the absorption of ^{65}Zn from an aqueous solution 5 groups of 10 mice were fastened for 20 hours, while 2 other groups of 10 mice had free access to the purified diet during this time. ^{65}Zn was administered by intragastric intubation ("i.g.") to one fastened and one non-fastened group (1.0 µCi ^{65}Zn/mouse), and in drinking water ("d.w.") to one fastened and one non-fastened group (on the average: 0.37 and 0.50 µCi ^{65}Zn/mouse, resp.), as described in exp. 1. No food was available during ^{65}Zn administration. To the third group of fastened mice ^{65}Zn was administered in drinking water (on the average: 0.61 µCi ^{65}Zn/mouse) with simultaneous availability of IRI-CB diet: "d.w. (+f.)". After 1.5 hrs the ^{65}Zn containing drinking water and the food were removed. These 5 groups received the IRI-CB diet again ad libitum 6 hours after ^{65}Zn administration until the end of the experiment. To the 2 remaining groups of fastened mice ^{65}Zn was again administered by intragastric intubation ("i.g.") (1.0 µCi/mouse), however, the IRI-CB diet was fed again ad libitum 20 hours, resp. 2 hours after ^{65}Zn administration until the end of the experiment.

Experiment 4 (see also Fig. 3). To test the influence of the type of diet on the absorption and retention of ^{65}Zn from an aqueous solution 4 groups of 10 mice, 4 weeks old, were not, as the other mice, adapted to the purified diet (IRI-CB), but fed the stock diet (SRMA) ad libitum during 2 weeks

and then fastened for 20 hours. ^{65}Zn was administered to 2 groups by intragastric intubation ("i.g.") and to 2 groups by intraperitoneal injection ("i.p."), as described in exp. 1 and 2 (1.0 and 0.3 µCi ^{65}Zn/mouse, resp.). The mice were fed again ad libitum 6 hours after ^{65}Zn administration, one group of each pair with the IRI-CB diet, the other group of each pair with the SRMA stock diet, until the end of the experiment.

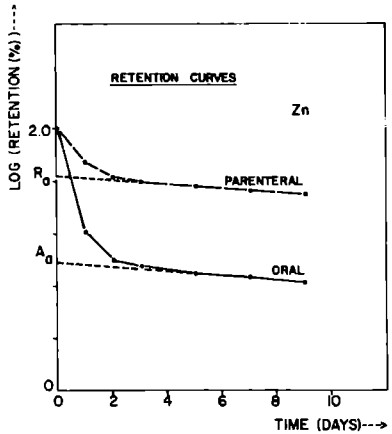


Fig. 1 Determination of the apparent absorption A_a and the apparent retention R_a, as in the method of Heth and Hoekstra [20].

The apparent absorption (A_a) of per os administered ^{65}Zn and the apparent retention (R_a) of parenterally administered ^{65}Zn were determined by whole-body counting during 9 days and extrapolation of the linear part of the semi-logarithmic retention curve to the ordinate, as described by Heth and Hoekstra [20] (Fig. 1). Mice which had consumed less than 0.2 µCi ^{65}Zn in drinking water or food were rejected. Mice which on basis of their ^{65}Zn retention on day 9 (R_9) were recognized as "outlier" according to Chauvenet's criterion [21] were not further included in the calculations.

Results

The results of experiment 1 (testing of 3 modes of oral administration) and experiment 2 (testing of 4 modes of parenteral administration) are presented in Fig. 2 (left part and right part, resp.). The apparent absorption of ^{65}Zn from an intubated aqueous solution, from the drinking water or from the food was 51%, 48% and 11%, resp. The apparent retention of ^{65}Zn injected i.p., s.c., i.m. or i.v. was 83-84%.

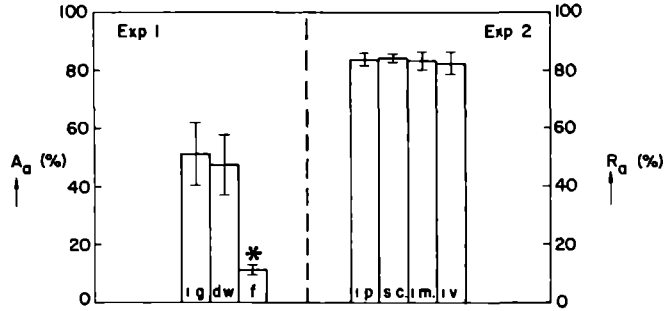


Fig. 2 Influence of the mode of administration on apparent absorption A_a and apparent retention R_a of ^{65}Zn. Left side: Oral administration. Right side: Parenteral administration. Abbr.: i.g.= intragastric intubation (1 µg Zn/ml); d.w.= drinking water (1 µg Zn/ml); f.= food (22 µg Zn/g); i.p.= intraperitoneally; s.c.= subcutaneously; i.m.= intramuscularly; i.v.= intravenously (1 µg Zn/ml, all).
* = significantly different by Student's t-test, $P<0.01$.

The results of experiment 3 (testing of the influence of food consumption) and experiment 4 (testing of the influence of the type of diet given) are presented in Fig. 3 (lower part and upper part, resp.). Food consumption prior to or following intragastric intubation of ^{65}Zn reduced the apparent absorption by 34% and 19%, resp., as compared to fasting. Food consumption prior to or during consumption of drinking water containing ^{65}Zn reduced the apparent absorption by 38% and 68%, resp., as compared to fasting. Consumption of stock diet instead of purified diet 6 hours after intubation or injection of ^{65}Zn reduced the apparent absorption by 32% and the apparent retention by 11%.

Discussion

The absorption of ^{65}Zn from an intubated solution was high and comparable to absorption of ^{65}Zn from drinking water, consumed on an empty stomach. The intubation mode, which is easy in operation and well reproducible as compared to administration in drinking water, may therefore serve as a simple model for drinking water consumption. The absorption of ^{65}Zn from food (IRI-CB purified diet) was only a fraction of the absorption found with the other two modes of administration. This may be the result of the higher zinc concentration of the food (22 µg/g) compared to the zinc concentration in the intubated solution and in the drinking water (1 µg/ml).

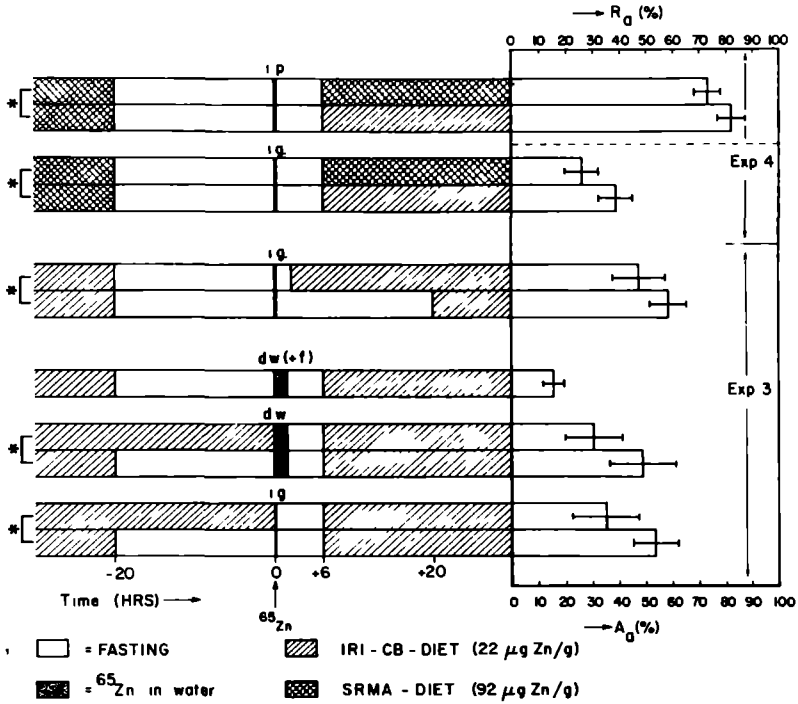


Fig. 3 Influence of food consumption and type of food (purified diet IRI-CB or stock diet SRMA) on apparent absorption A_a and apparent retention R_a of ^{65}Zn. Abbr.: i.g.= intragastric intubation; d.w.= drinking water; (+f.)= simultaneous food consumption; i.p.= intraperitoneal injection.
* = significantly different by Student's t-test, $P < 0.05$.

Comparison of four modes of parenteral administration showed no influence of the site of injection on the apparent retention of ^{65}Zn. Apparently in all cases the injected zinc was easily picked up from the injection site and distributed over the body. It must be pointed out that the observations were made over a period of days. Observations within hours after injection may reveal temporary differences in distribution and excretion patterns of injected ^{65}Zn.

Food consumption within 20 hours before or after ^{65}Zn administration

generally resulted in a reduction of the apparent absorption. Simultaneous consumption of food and ^{65}Zn containing drinking water reduced A_a almost to the level of ^{65}Zn absorption from food, indicating a significant mixing of food and drinking water in the upper gastro-intestinal tract. These findings are in agreement with those of Quarterman and Morrison [22], who showed that the absorption of Pb, Hg, Fe, Cu, Zn and Ca was 2- to 10-fold higher in rats fastened 16-24 hrs than in rats fastened 0-12 hrs. The reduction of A_a by food consumption prior to and during ^{65}Zn administration and the low absorption of ^{65}Zn from food itself is probably due to isotope dilution with zinc from the food and to binding of ^{65}Zn to undigested food components. The reduction of A_a by food consumption following ^{65}Zn administration may be due to an accelerated intestinal passage of ^{65}Zn. Another reason may be a stimulation of the pancreas by consumed food. The secretion of zinc containing digestive enzymes by the pancreas may increase the excretion in the intestine of just absorbed ^{65}Zn, resulting in a lower apparent absorption.

The apparent absorption and retention of ^{65}Zn were also influenced by the type of diet given. When stock diet was fed before ^{65}Zn administration, A_a was significantly lower than after adaptation to the purified diet (see exp. 3). When stock diet was fed 6 hours after ^{65}Zn administration, both A_a and R_a were reduced. The high zinc content of the stock diet may play a role by increasing the rate of turnover of zinc. Furthermore phytate and fiber in the stock diet may prevent reabsorption of endogenous ^{65}Zn, excreted in the intestine.

Every possible interference between ^{65}Zn and food seems to reduce the apparent absorption. The consequence of this food effect is that in animal studies of zinc absorption using isotopes the mode of isotope administration and conditions of food consumption must be very carefully chosen to avoid undesired influences of the food; they should also be described extensively and accurately in the "Methods"-section of publications in order to allow reproducibility of results and comparison to results of others.

The observed difference between zinc absorption from drinking water and that from food and the effect of food consumption on zinc absorption from water may have implications for the estimation of human uptake of trace elements from the diet. It is already recognized [23], that bioavailability of trace elements is generally larger in drinking water than in food, as is confirmed by these results with zinc. Moreover, not only consumed quantities of water and food and the bioavailabilities from these sources are important, but also the consumption pattern which determines the degree of interference of both and thus the final absorption. For zinc the drinking water contribution is low [23], so the degree of food/drinking water interaction will not strongly influence the estimated uptake. For trace elements with a higher contribution from the drinking water to the total uptake, an estimation tak-

ing in account the consumption pattern and food/drinking water interactions may be lower than an estimation only on basis of concentrations and bioavailability in water and food.

A second application of the described results can be found in medical science. When trace elements are administered for therapeutic purposes, an optimal choice of the conditions of administration is important to achieve maximum availability. Zinc should be given as a solution in a highly available form and consumed on an empty stomach, preferably some time before a meal. However, this mode of administration may be accompanied by undesired side-effects as nausea, which may restrict the dose.

Our results also confirm the importance of standard administration conditions in diagnostical investigation of human zinc metabolism. Uncontrolled food consumption of human volunteers or patients may lead to irreproducible and divergent results, as was already noticed by Molokhia et al. [10]. Aamodt et al. [7] indicated that intercomparison of human zinc absorption studies is difficult due to the different fasting conditions used. A standard protocol for zinc absorption studies using isotopes can give a solution to this problem.

At least some of the observed effects also occur with other trace elements [22]. It can be expected that the results, described for Zn, are founded on more general priciples. However, further research on this subject is needed.

Acknowledgements

Prof. J.P.W. Houtman and Dr. J.H.M. Van Tongeren are acknowledged for critical review of the manuscript. Ms. H.K. Terlouw is thanked for technical assistance.

References

1. Weigand E, Kirchgessner M. Total true efficiency of zinc utilization: Determination and homeostatic dependence upon the zinc supply status in young rats. J Nutr 1980; 110: 469-80
2. Franz KB, Kennedy BM, Fellers DA. Relative bioavailability of zinc using weight gain of rats. J Nutr 1980; 110: 2263-71.
3. Jackson MJ, Jones DA, Edwards RHT. Zinc absorption in the rat. Br J Nutr 1981; 46: 15-27.
4. Davies NT. Studies on the absorption of zinc by rat intestine. Br J Nutr 1980; 43: 189-203.
5. Jain RK, Gerlowski LE, Weissbrod JM, Wang J, Pierson Jr. RN Kinetics of uptake, distribution, and excretion of zinc in rats. Ann Biomed Engin 1981; 9: 347-61.

6. Arvidsson B, Cederblad Å, Björn-Rasmussen E, Sandström B. A radionuclide technique for studies of zinc absorption in man. Int J Nucl Med Biol 1978, 5: 104-9.
7. Aamodt RL, Rumble WF, Johnston GS, Markley EJ, Henkin RI Absorption of orally administered ^{65}Zn by normal human subjects. Am J Clin Nutr 1981; 34: 2648-52.
8. Payton KB, Flanagan PR, Stinson EA, Chodirker DP, Chamberlain MJ, Valberg LS. Technique for determination of human zinc absorption from measurement of radioactivity in a fecal sample or the body. Gastroenterology 1982; 83. 1264-70.
9. Aamodt RL, Rumble WF, Johnston GS, Foster D, Henkin RI. Zinc metabolism in humans after oral and intravenous administration of Zn-69m Am J Clin Nutr 1979; 32. 559-69.
10. Molokhia M, Sturniolo G, Shields R, Turnberg LA. A simple method for measuring zinc absorption in man using a short-lived isotope (^{69m}Zn). Am J Clin Nutr 1980; 33· 881-6.
11. Janghorbani M, Ting BTG, Young VR. Accurate analysis of stable isotope ^{68}Zn, ^{70}Zn and ^{58}Fe in human feces with neutron activation analysis. Clin Chim Acta 1980; 108: 9-24.
12. Janghorbani M, Ting BTG, Young VR, Steinke FH. Intrinsic labelling of chicken meat with stable isotopes of zinc, for intended use in human feeding studies: feasibility and design considerations. Br J Nutr 1981; 46: 395-402.
13 Turnlund JR, Michel MC, Keyes WR, King JC, Margen S. Use of enriched stable isotopes to determine zinc and iron absorption in elderly men. Am J Clin Nutr 1982; 35: 1033-40.
14. Solomons NW, Janghorbani M, Ting BTG, Steinke FH, Christensen M, Bijlani R, Istfan N, Young VR. Bioavailability of zinc from a diet based on isolated soy protein: Application in young men of the stable isotope tracer, ^{70}Zn. J Nutr 1982; 112: 1809-21.
15 Janghorbani M, Istfan NW, Pagounes JO, Steinke FH, Young VR. Absorption of dietary zinc in man. comparison of intrinsic and extrinsic labels using a tripel stable isotope method. Am J Clin Nutr 1982; 36 537-45.
16. Lo GS, Steinke FH, Ting BTG, Janghorbani M, Young VR Comparative measurement of zinc absorption in rats with stable isotope ^{70}Zn and radioisotope ^{65}Zn. J Nutr 1981; 111: 2236-9.
17 Gordon EF, Gordon RC, Passal DB. Zinc metabolism: Basic, clinical, and behavioral aspects. J Pediatr 1981; 99. 341-9.
18. Solomons NW. Biological availability of zinc in humans. Am J Clin Nutr 1982, 35: 1048-75.
19. Van Barneveld AA, Morse H. A new diet for trace element research with rats and mice. Z Versuchstierk 1982; 24: 24.
20. Heth DA, Hoekstra WG. Zinc-65 absorption and turnover in rats. I. A pro-

cedure to determine zinc-65 absorption and the antagonistic effect of calcium in a practical diet. J Nutr 1965; 85: 367-74.

21. De Soete D, Gijbels R, Hoste J. In: Neutron activation analysis, pp. 506-7. Chemical Analysis, Vol 34 (Eds Elving PJ, Kolthoff JM). London, UK: Wiley-Interscience.
22. Quarterman J, Morrison E. The effects of short periods of fasting on the absorption of heavy metals. Br J Nutr 1981; 46: 277-87.
23. Commins BT. The contribution drinking water makes to man's overall exposure to inorganic substances. Proc Int Conf Heavy Metals in the Environment, Amsterdam 1981, 35-8. Edinburgh, UK: CEP Consultants Ltd, 1981.

4.5. PAPER IV

INFLUENCE OF TRACE ELEMENT CONCENTRATION ON ABSORPTION AND RETENTION OF TRACE ELEMENT RADIOTRACERS IN MICE: A STUDY WITH ^{75}SE, ^{64}CU, ^{65}ZN AND ^{203}PB

A.A. Van Barneveld and C.J.A. Van den Hamer

ABSTRACT The absorption and retention of trace element radioisotopes, administered to mice by intragastric intubation and by intraperitoneal injection, may depend on the concentration of the trace element in the administered solution. In a study with ^{75}Se (as selenite) and with ^{64}Cu, ^{65}Zn and ^{203}Pb (as cation) the element concentration in the dose was varied: 0.2-25 µg/ml (Se), 0.2-100 µg/ml (Cu), 1.0-100 µg/ml (Zn) and 0.2-125 µg/ml (Pb). The true absorption of ^{64}Cu and ^{75}Se decreased with increasing element concentration from 26% to 8% and from 95% to 88%, resp. The true absorption of ^{65}Zn and ^{203}Pb were relatively constant (60% both), but ^{203}Pb absorption decreased strongly at the highest concentration. Histidine, added to Zn in a 10-fold excess on a molar basis, did not ameliorate the absorption of intubated ^{65}Zn. Measurement of ^{65}Zn absorption from food showed a strong decrease in true absorption (from 53% to 19%) when food Zn increased from 1.5 to 100 µg/g. Possible explanations for this food effect are discussed. The retention of intraperitoneally injected ^{64}Cu, ^{65}Zn and ^{75}Se decreased from 47% to 21%, from 85% to 78%, and from 65% to 22%, resp. The retention of ^{203}Pb increased from 82% to 105%, which was probably caused by the formation of inclusion particles in peritoneal macrophages at higher doses of injected Pb. Whole-body autoradiography of ^{203}Pb injected intravenously in a rat showed incorporation of Pb in bone and kidneys and accumulation of Pb in the caecum and colon due to endogenous excretion. The results indicate that the concentration of the trace element in the dose may influence the absorption and retention of ^{75}Se, ^{64}Cu, ^{65}Zn and ^{203}Pb by various physiological mechanisms.

Introduction

In the last 10 years many studies reported data on the absorption and retention of trace elements. The use of radioactive and stable isotopes in these studies has proven to be very successful. Little is known, however, about the relation between absorption and retention of the tracer on the one hand and the concentration of its carrier in the administered dose on the other hand. This hampers intercomparison of the various results, because the concentrations used in different investigations are usually not the same. Intercomparison therefore demands knowledge of this relationship. In the present study the concentration dependence of the absorption and retention

was investigated in mice for four elements. One (Se) was in the anionic form (SeO_3^{2-}), the others in the cationic form (Cu^{2+}, Zn^{2+} and Pb^{2+}). As a measure for absorption and retention processes the apparent and true absorption and the apparent retention were used as described by Heth and Hoekstra [1]. This study may provide more insight in the role of the concentration factor, especially in relation to isotope dilution effects, and may also contribute to the understanding of the homeostatic regulation of trace elements in the body.

Materials and methods

Diets. A standard purified diet (IRI-CB diet; CB= casein based) and a Zn-poor variant (IRI-OBLZ diet; OBLZ= ovalbumin based low Zn) were obtained from Hope Farms, Woerden, The Netherlands. The composition of the IRI-CB purified diet was reported earlier [2]. According to analysis by atomic absorption spectrometry it contained 22 µg Zn/g. Casein as the source of protein in the IRI-CB diet was replaced by ovalbumin in the IRI-OBLZ variant, dl methionine supplementation, necessary only when casein is used, was omitted; zinc oxide was omitted from the trace element premix. The resulting IRI-OBLZ diet contained 1.5 µg Zn/g.

Radioisotopes. Carrier-free ^{65}Zn was obtained from The Radiochemical Centre, Amersham, UK. ^{75}Se (356 Ci/g) and carrier-free ^{203}Pb were obtained from New England Nuclear, Boston, Mass., USA. ^{64}Cu (1 Ci/g) was prepared in the nuclear reactor of the Interuniversity Reactor Institute, Delft, The Netherlands.

Animals. Female Swiss Random mice were obtained from the Central Institute for the Breeding of Laboratory Animals-TNO, Austerlitz, The Netherlands. After arrival, the mice, 4 weeks old, were housed in macrolon cages with stainless steel lids and glass drinking bottles. They were fed the standard purified diet and received demineralized drinking water ad libitum during 2 weeks. The need for this adaptation period is discussed elsewhere [2]. After this period the mice were distributed at random in groups of 10 animals, housed in metabolic cages and fastened for 20 hours. After isotope administration they were fastened another 20 hours and then the standard purified diet was fed again ad libitum until the end of the experiment.

Radioisotope administration. Solutions of Se (as sodium selenite) and of Cu, Zn and Pb (as the chloride salts), radiotracered with ^{75}Se, ^{64}Cu, ^{65}Zn and ^{203}Pb, resp., were prepared in demineralized water (for intragastric administration) or in acetate buffer, 0.05 M, pH 5.6, 0.7% NaCl (for intraperitoneal injection). The trace element concentrations in both types of solutions were varied: 0.2, 1, 5 and 25 µg Se/ml, 0.2, 1, 5, 20 and 100 µg Cu/ml; 1, 5, 20 and 100 µg Zn/ml; 0.2, 1, 5, 25 and 125 µg Pb/ml. Each radiotracered solution was administered to a group of 10 fastened mice by

Table 1 Comparison of the range of administered doses (in 0.3 ml) and the estimated daily intake of trace elements (Se, Cu, Zn, Pb) in the mice

Element	Range	Daily intake [1]
	µg	µg
Se	0.06 - 7.5	0.45
Cu	0.06 - 30.0	30
Zn	0.3 - 30 0	60
Pb	0.06 - 37.5	-

[1] Assuming a daily consumption of 3 gram of IRI-CB food

intragastric intubation ("i.g.", 0.3 ml aqueous solution/mouse) or by intraperitoneal injection ("i.p.", 0.3 ml acetate buffer/mouse) The administered quantities of ^{75}Se, ^{65}Zn and ^{203}Pb were 1 µCi/mouse, for the 5 concentrations of Cu: 0.06, 0.3, 1.5, 1.5 and 1.5 µCi ^{64}Cu/mouse, respectively

The intragastric intubation of 4 concentrations of Zn was also carried out after addition of histidine, as an easily absorbed Zn carrier, to the Zn solution at a molar ratio of histidine/Zn=10 Another experiment was carried out using food as the Zn carrier. Portions of 10 g of the Zn-poor purified diet (IRI-OBLZ diet containing 1.5 µg Zn/g) were supplemented with increasing amounts of zinc chloride (final zinc concentrations: 1.5, 5, 20 and 100 µg Zn/g) and added to 2 ml of carrier-free ^{65}Zn solution and mixed thoroughly. Also a 10 g portion of standard purified diet (IRI-CB diet containing 22 µg Zn/g) was mixed with 2 ml ^{65}Zn solution These 5 portions were made available to 5 groups of 10 mice and consumed within 1.5 hours The consumed dose in each mouse was measured immediately afterwards by whole-body counting

Measurements. Retention of the administered radioisotopes was measured by whole-body counting over a period of 3-9 days depending on the half-life of the isotopes. For whole-body counting two well-type NaI-crystals were used, as described earlier [2] On the last day the mice were sacrificed and from the mice used in the ^{203}Pb experiment some organs (liver, kidneys and gastrointestinal tissue) were collected and its ^{203}Pb content was measured in an auto-gamma scintillation spectrometer (Packard 5120) Mice which on basis of their isotope retention on day 9 (R_9) were recognized as "outlier" according to Chauvenet's criterion [3] were not further included in the calculations. Faeces and urine were collected and measured separately in the Packard spectrometer. ^{203}Pb in the urine was partly lost because of unnoticed precipitation in the collection tubes, from which the urine was decanted before counting. Here the urine contribution was calculated from the balance data.

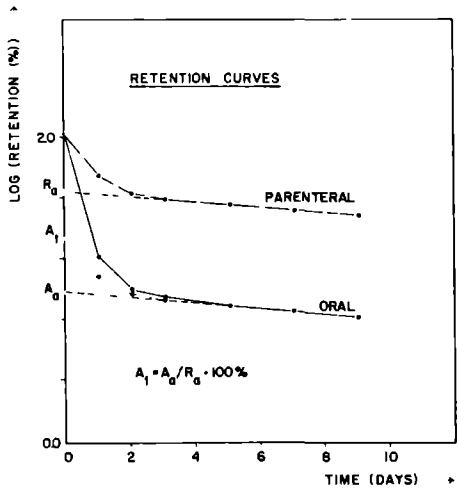


Fig. 1. Determination of apparent absorption A_a, apparent retention R_a and true absorption A_t from the retention curves as in the method of Heth and Hoekstra [1]. Typical retention curves of Zn are shown as example.

Apparent absorption A_a and apparent retention R_a were calculated as in the method of Heth and Hoekstra [1] by extrapolation of the linear part of the semi-logarithmic retention curve to time zero and determination of the intersection point with the ordinate (A_a for orally administered isotopes and R_a for parenterally administered isotopes) (Fig. 1). For ^{64}Cu the measurement of a complete retention curve was impossible because of its short half-life ($t_{1/2}$=12,8 h). The retention on the third day (R_3) was used as an approximation of A_a and R_a ("A3" and "R3", resp.).

Calculation of the true absorption. The true absorption A_t was calculated with the formula of Heth and Hoekstra $A_t=(A_a/R_a) \times 100\%$ [1] (Fig. 1). This formula assumes an independence of R_a on the concentration of metal injected. This appeared, however, not always true. Therefore A_t was corrected for the concentration dependance of R_a (see Appendix).

Whole-body autoradiography. Carrier-free ^{203}Pb (200 µCi) was injected intravenously in a male Wistar rat (200 g). After 16 hours the rat was killed by CO_2-inhalation and deep-frozen in 3% carboxymethyl cellulose according to Larsson and Ullberg [4]. Coupes of 20 µm were made with a whole-body cryomicrotome (LKB-PMV-2258) and autoradiograms were made by use of Structurix D10 X-Ray film (Agfa-Gevaert), using an exposure time of 24 hrs.

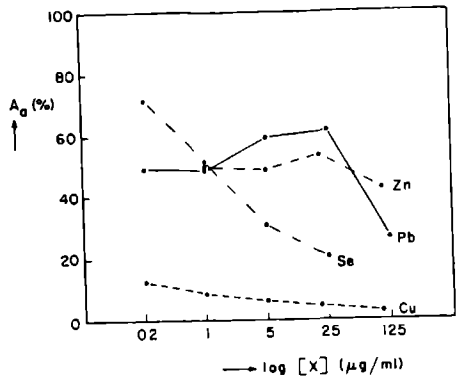


Fig. 2 The apparent absorption A_a of ^{75}Se, ^{64}Cu, ^{65}Zn and ^{203}Pb as a function of the element concentration in the intragastrically intubated solution.

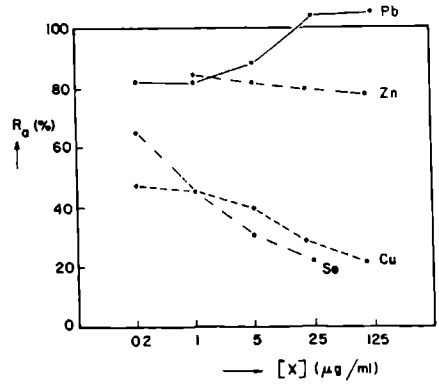


Fig. 3 The apparent retention R_a of ^{75}Se, ^{64}Cu, ^{65}Zn and ^{203}Pb as a function of the element concentration in the intraperitoneally injected solution.

Results

The apparent absorption A_a, apparent retention R_a and true absorption A_t of ^{75}Se, ^{64}Cu, ^{65}Zn and ^{203}Pb were measured for concentration ranges of the trace elements more or less corresponding to the normal daily intake (Table 1). The results (Fig. 2-5) show a striking diversity among the four elements:

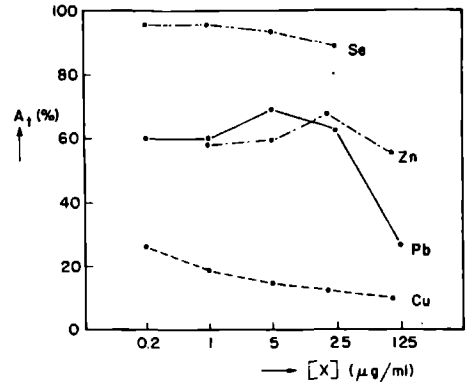


Fig. 4 The true absorption A_t of ^{75}Se, ^{64}Cu, ^{65}Zn and ^{203}Pb as a function of the element concentration in the intragastrically intubated solution.

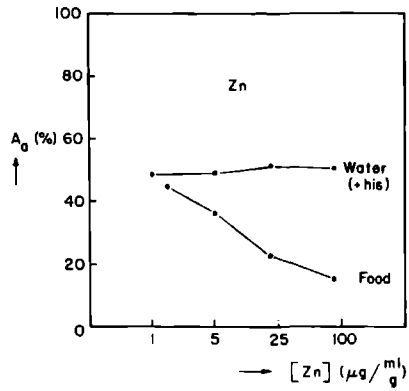


Fig. 5 The apparent absorption A_a of ^{65}Zn from a solution containing histidine in a molar ratio of histidine/zinc=10 and from food (IRI-OBLZ diet) as a function of the element concentration in the intragastrically intubated solution (µg Zn/ml) and in the food (µg Zn/g), respectively.

Selenium. Both the apparent absorption A_a (Fig. 2) of intubated ^{75}Se and the apparent retention R_a (Fig. 3) of injected ^{75}Se showed a strong concentration dependence, but were approximately equal for each concentration. This indicates that the true absorption of selenite in the gut was close to

100%. Calculation of the true absorption A_t according to Heth and Hoekstra showed values higher than 100% (Table 2), which probably was the result of the limited accuracy of the measurements (see standard deviations of A_a and R_a in Table 2). This limited accuracy can also be seen from the balance data presented in Fig. 6. The sum of the faecal and urinary excretions during the first 4 days after dosage (F_{1-4} and U_{1-4}, resp.) was somewhat higher than the decrease in retention (100 - R_4) during this period. Fig. 6 also shows that the excretion in the urine played a major role in homeostatic control. The faecal contribution to the excretion was somewhat higher after oral administration than after injection. Recalculation of the true absorption by the assumption that the excess of ^{75}Se in the faeces after oral administration (F_{ex}) originated from unabsorbed selenite showed that A_t was actually somewhat lower than 100%, specially for the higher concentrations (Table 2; Fig. 4).

Copper. A strong concentration dependence both of A_a and of R_a was found; A_t decreased strongly with increasing Cu concentration (Table 2). Note that correction of A_t for the concentration dependence of R_a had a strong effect. For Cu this correction is therefore essential. Fig. 7 shows that Cu was excreted mainly in the faeces, also after parenteral administration. The sum of faecal and urinary excretions during the first 3 days after dosage (F_{1-3} and U_{1-3}, resp.) was almost equal to the decrease of the retention (100 - R_3) during this period.

Zinc. No concentration dependence was found for A_a, but R_a decreased slightly; A_t was constant (about 60%) (Table 2). Addition of histidine in a 10-fold molar excess over Zn did not ameliorate ^{65}Zn absorption (Table 3). A practically identical curve as found without histidine was observed (Fig. 5). The smoothness of the curve suggests that the irregularity of the curve observed in absence of histidine (Fig. 2) could have been the result of the relative inaccuracy of the measurement of A_a.

Fig. 5 also shows ^{65}Zn absorption from food. Zn absorption from the Zn-deficient diet IRI-08L2 containing 1.5 µg Zn/g as an intrinsic part of its basic components was approximately equal to Zn absorption from a solution, but sharply decreased when the Zn concentration in the diet was raised upto 100 ug/g by supplementation with ZnCl2 (Table 3). Absorption of Zn from the standard purified diet IRI-C8 containing 22 µg Zn/g was not significantly different from that of the IRI-08L2 diet adjusted to 20 µg Zn/g. The type of protein in the diets (ovalbumin and casein, resp.) therefore had no strong effect on the Zn absorption.

Lead. A_a showed a slight increase with increasing Pb concentrations, but fell off at the highest Pb concentration; R_a showed an increase from 82% to calculated values higher than 100%; A_t was constant (about 60%) up to 25 µg/ml (Table 2). Fig. 8 shows that Pb was excreted both in faeces and in urine. The excretion of injected Pb, particularly in the urine, appeared to

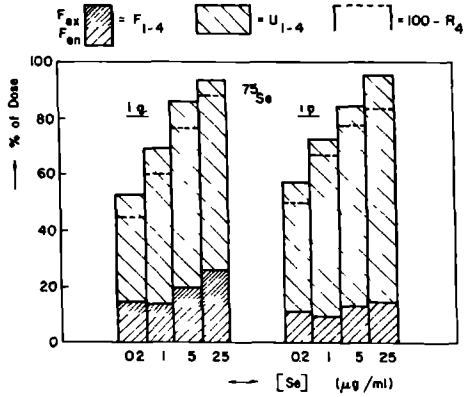


Fig. 6 The distribution of ^{75}Se, excreted during the first 4 days, over faeces (F_{1-4}) and urine (U_{1-4}), and the decrease of the retention during this period (100 - R_4), as a function of the element concentration in the intragastrically intubated or intraperitoneally injected solution. A distinction has been made between excretion with the faeces of endogenous Se (F_{en}) and of exogenous (unabsorbed) Se (F_{ex}). For this it was assumed that absorbed ^{75}Se was excreted in faeces and urine to a similar extent as injected ^{75}Se.

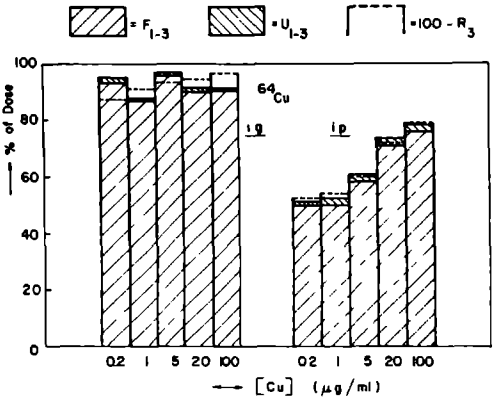


Fig. 7 The distribution of ^{64}Cu, excreted during the first 3 days, over faeces (F_{1-3}) and urine (U_{1-3}), and the decrease of the retention during this period (100 - R_3), as a function of the element concentration in the intragastrically intubated or intraperitoneally injected solution.

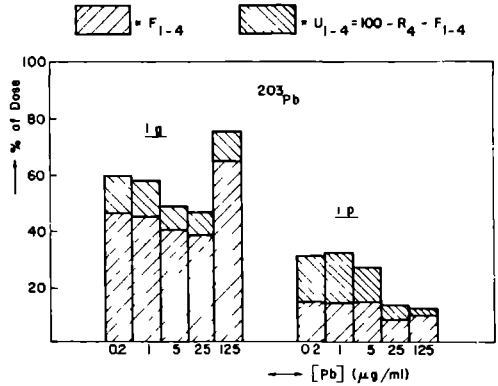


Fig. 8 The distribution of ^{203}Pb, excreted during the first 4 days, over faeces (F_{1-4}) and urine (U_{1-4}) as a function of the element concentration in the intragastrically intubated or intraperitoneally injected solution. U_{1-4} was not measured, but calculated from the difference between the decrease of the retention (100 - R_4) and the faecal excretion (F_{1-4}) during this period.

be strongly reduced at higher doses, which could indicate a limited excretion capacity of the kidneys. Investigation of some organs of the mice injected intraperitoneally with ^{203}Pb showed a strong retention of ^{203}Pb in the gastrointestinal tissue (including the mesentery) at higher doses (Fig. 9).

Pb was mainly incorporated into bone and kidneys, as is demonstrated by autoradiograms of a rat sacrificed 16 hours after intravenous injection of ^{203}Pb (Fig. 10). In these autoradiograms ^{203}Pb activity can also be seen in the caecum and colon, indicating endogenous faecal excretion of ^{203}Pb.

Discussion

The apparent absorption of Se was much lower and showed a much stronger concentration dependence than the true absorption of Se, which was almost complete at all concentrations. The ultimate retention of Se appeared to be strongly regulated through the urinary excretion. These results are very well in agreement with those found for rats. Thomson and Stewart [5] found a true absorption of 92% for selenite, and Burk et al. [6] found for intraperitoneally injected selenite a decrease of the apparent retention from 100% to 14%, when the Se concentration increased from 0.005 to 200 µg/ml. A similar tendency was observed in a limited experiment with mice by Hansen and Kristensen [7]. Also in these studies [6,7], the retention of Se was found to be

Table 2 The concentration dependence of the apparent absorption A_a, the apparent retention R_a and the true absorption A_t of ^{75}Se, ^{64}Cu, ^{65}Zn and ^{203}Pb. [1]

[X]	n	A_a [2]	n	R_a [2]	A_t [3]	A_t' [4]	A_t'' [5]
µg/ml				%			
[Se]							
0.2	10	71 ± 7 [a]	10	65 ± 6 [p]	109	112	95
1	10	52 ± 6 [b]	9	46 ± 2 [q]	113	117	95
5	10	31 ± 3 [c]	10	31 ± 3 [r]	102	102	93
25	9	21 ± 2 [d]	10	22 ± 2 [s]	94	92	88
[Cu] [6]							
0.2	9	12.6 ± 3.2 [a]	9	47 ± 5 [p]	27	26	
1	10	8.6 ± 1.6 [b]	10	46 ± 3 [p]	19	18	
5	10	6.4 ± 2.1 [b]	10	40 ± 3 [q]	16	14	
20	9	4.8 ± 1.0 [c]	10	28 ± 4 [r]	17	11	
100	10	3.0 ± 0.7 [d]	10	21 ± 2 [s]	14	8	
[Zn]							
1	10	49 ± 10 [a]	6	85 ± 3 [p]	58	58	
5	9	49 ± 11 [a]	8	82 ± 2 [q]	60	59	
20	10	54 ± 16 [a]	8	80 ± 4 [qr]	67	67	
100	9	43 ± 10 [a]	7	78 ± 4 [r]	57	54	
[Pb]							
0.2	9	49 ± 6 [b]	10	82 ± 4 [p]	60	60	
1	10	49 ±14 [b]	10	82 ± 5 [p]	59	59	
5	10	59 ± 8 [c]	10	88 ± 4 [q]	67	68	
25	10	61 ± 9 [c]	9	104 ± 3 [r]	59	62	
125	9	26 ± 6 [a]	9	105 ± 4 [r]	25	25	

[1] Data are given as mean ± SD for n mice.

[2] Means with common superscripts are not significantly different by Student's t-test, $P < 0.05$.

[3] $A_t = (A_a/R_a) \times 100\%$.

[4] A_t' = A_t after correction for dose dependence of R_a.

[5] $A_t'' = 100\% - F_{ex}$, in which F_{ex} is the (exogenous) faecal excretion of unabsorbed Se.

[6] A_a and R_a are approximated by R_3.

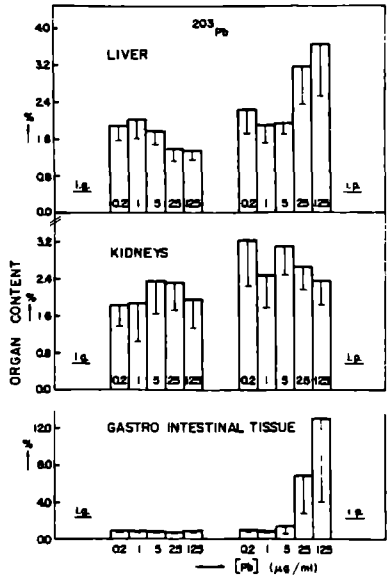


Fig. 9 ^{203}Pb content in liver, kidneys and gastrointestinal tissue (including the mesentery) of mice, sacrificed on day 9 after intragastric intubation (i.g.) or intraperitoneal injection (i.p.) of ^{203}Pb. The ^{203}Pb content is expressed as % of the total body content of ^{203}Pb on the day of sacrifice (R9).

primarily regulated by the urinary excretion. At higher doses excretion of Se may partly occur by exhalation as dimethylselenide [8]. The balance data (Fig. 6) indicated, however, that in the present experiments no appreciable amount of ^{75}Se could have been lost this way.

Little is known about a possible concentration dependence of Cu absorption and retention in rats and mice. From work of Gitlin with mice [9] it can be estimated that the apparent absorption of an oral Cu dose in water decreased from 16% to 11% when the dose increased from 11 to 95 µg Cu. A similar dependence has been found in rat intestine for Cu doses increasing from 1 to 100 µg [10]. Our results (Fig. 2) support these data. Not only absorption, however, but also excretion of Cu appeared to be regulated; the latter primarily by endogenous faecal excretion (Fig. 7).

No indication was found for regulation of Zn absorption from an intubated solution in the concentration range of 1 to 100 µg/ml. According to Davies [11] the absorption mechanism of Zn in rats is saturated at intraluminal Zn

Table 3 The concentration dependence of the apparent absorption A_a and the true absorption A_t of ^{65}Zn, administered in combination with histidine at a molar ratio of His/Zn=10, and of ^{65}Zn, administered as a mixture with food. [1]

Histidine				Food			
[Zn]	n	A_a [2]	A_t [3]	[Zn]	n	A_a [2]	A_t [3]
µg/ml		%	%	µg/g		%	%
1	12	49 ± 8 [a]	58	1.5 [4]	7	45 ± 7 [a]	53
5	10	49 ± 6 [a]	60	5 [4]	9	37 ± 8 [b]	45
20	11	51 ± 7 [a]	64	20 [4]	9	23 ± 4 [c]	29
100	11	51 ± 7 [a]	65	100 [4]	9	15 ± 4 [d]	19
				22 [5]	9	23 ± 5 [c]	29

[1, 2, 3] As in Table 2.

[4] Zn-adjusted IRI-OBLZ purified diet.

[5] Standard IRI-CB purified diet.

concentrations above 50 µg/ml. The results of Smith and Cousins [12] suggest that in rats saturation takes place at intraluminal Zn concentrations above 100 µg/ml, whereas Jackson et al. [13] have found that Zn absorption from an intubated solution decreases at Zn concentrations above 130 µg/ml. In man a Zn absorption of about 55% was found for doses of 100 ml drinking water containing 11.5 to 115 µg Zn/ml; at higher concentrations, viz., 288 and 576 µg/ml, the absorption decreased to 40% and 25%, resp. [14]. On basis of these data we may assume that our curves of the apparent and true absorption of ^{65}Zn should have bent down, if Zn concentrations higher than 100 µg/ml had been applied.

In experiments with rats [15] and chicks [16] the absorption of Pb from an intubated solution was independent of the concentration up to 10 µg Pb/ml, but strongly decreased at higher concentrations. In the present experiment with mice the threshold was somewhat higher (25 µg Pb/ml). In vitro experiments with everted rat intestine [17] indicated a limited absorption capacity of the intestine for Pb. The strong retention of intraperitoneally injected ^{203}Pb in the gastrointestinal tissue (including the mesentery) at higher Pb doses could have been the result of inclusion of Pb in peritoneal macrophages, as has been described by Wapnir et al. [18]. In this way Pb could have become temporarily unavailable for uptake from the peritoneal cavity, which could explain the increase of the apparent retention of Pb up to

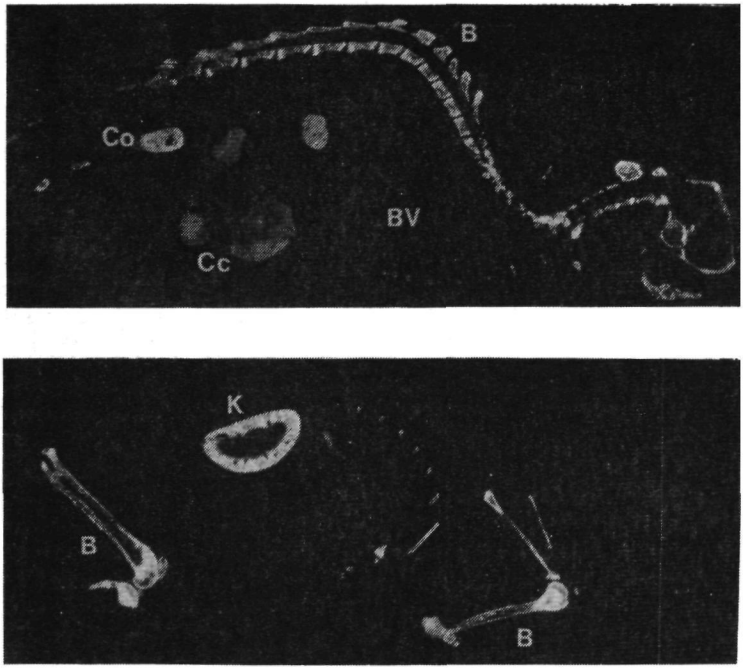


Fig. 10 Whole-body autoradiogram of ^{203}Pb, injected intravenously in a male Wistar rat. Accumulation of ^{203}Pb is seen in bone (B) and kidneys (K). Caecum (Cc) and colon (Co) contain ^{203}Pb originating from endogenous excretion. Large blood vessels (BV) in the liver are seen as dark spots, indicating negligible ^{203}Pb binding in the blood.

calculated values above 100%.

The absence of an effect of addition of histidine as a carrier for Zn on ^{65}Zn absorption from water suggests that Zn in water without histidine already has an optimal availability. The true absorption of about 60%, observed for Zn at concentrations ranging from 1 to 100 µg/ml, is obviously a maximum for mice under the present conditions. This maximum is not determined by the absorption capacity, because this capacity does not seem to be a limiting factor in the concentration range investigated. The reason for this incomplete absorption of Zn at all concentrations is not understood.

The results with histidine contrast with those obtained for the ^{65}Zn absorption from food (Fig. 5), which show a strong concentration dependence

in the same range. The question is why this difference in concentration dependence between Zn absorption from water and from food exists and what are the causing mechanisms. One explanation could be that Zn in water is immediately available and is absorbed more proximally in the intestine, whereas Zn in food becomes available during digestion and is absorbed more distally in the intestine. If different absorption mechanisms exist at different sites, this could cause the observed difference in concentration dependence.

Another explanation could be that, as Evans et al. [19] have suggested, the pancreatic juice may contain a zinc binding ligand (ZBL), which promotes Zn absorption by transferring Zn from the food components to the mucosal cell. Such a ZBL could counteract the binding of Zn to undigested food components. Probably being limited in quantity, it would primarily be effective at lower Zn concentrations in the food. At higher Zn concentrations the binding to food components would dominate, reducing the Zn availability. It has been suggested that the bile instead of the pancreatic juice is the source of a ZBL [20]. We like to point out that a ZBL could also originate from food itself during digestion. For example amino acids, oligosaccharides or fatty acids might play such a role. The amount of ZBL would be proportional to the amount of food consumed and its effect limited at higher Zn concentrations of the food.

The results obtained on the ^{65}Zn absorption from an intubated solution and from food could also be of interest in view of the isotope dilution hypothesis of Evans et al. [21]. They suggested that the absorption of orally administered ^{65}Zn may be reduced by dilution of ^{65}Zn with endogenous zinc excreted in the intestine. Flanagan et al. [22] referred to this hypothesis to explain the higher absorption of ^{65}Zn from food at lower Zn concentrations in zinc-deficient mice as compared to zinc-replete mice. Assuming that in the present experiments isotope dilution by endogenous excretion of Zn was negligible because of the adequate but marginal Zn supply from the diet (22 µg Zn/g), our results seem to indicate that in mice ^{65}Zn absorption from food should be more sensitive to isotope dilution than ^{65}Zn absorption from water; this, because ^{65}Zn absorption from food shows a concentration dependence for practical doses, whereas ^{65}Zn absorption from water only shows such dependence at much higher doses.

Acknowledgements

Prof. J.P.W. Houtman and Dr. J.H.M. Van Tongeren are acknowledged for critical review of the manuscript. Mr. L. Prinsen is thanked for whole-body autoradiography of ^{203}Pb in the rat. Mr. T.M.W. Stolk and Mr. C.A. Witteman are thanked for technical assistance.

Appendix. Description of the method used for correction of A_t in case of a concentration dependence of R_a.

The method is explained using the following example:

Assume A_t is to be calculated for Cu, administered orally in a solution containing 20 µg Cu/ml. For [Cu]= 20 µg/ml it was found that:

$A_a(20)$ = 4.8% and $R_a(20)$ = 28%.

According to the formula $A_t = (A_a/R_a) \times 100\%$ (Fig. 1), $A_t(20)$ = 17%.

If, however, the true absorption of Cu should be 17%, then the retention of the absorbed Cu should correspond to the retention of an injected dose, containing 17% of 20 µg Cu/ml. For such a dose the apparent retention R_a is not the value found for an injected dose of 20 µg Cu/ml ($R_a(20)$), but the value found for an injected dose of 0.17x20=3.4 µg Cu/ml ($R_a(3.4)$). This new value, say R_a', can be estimated by interpolation between $R_a(1.0)$ and $R_a(5.0)$ (Fig. 3) and is about 41%.

When this R_a' is used for calculation of A_t, a new value is found, say A_t' :

$A_t' = (A_a(20) / R_a') \times 100\% = 11.7\%$.

It can now again be said, that the retention of the absorbed Cu should correspond to the retention of an injected dose, containing 11.7% of 20 µg Cu/ml (= 2.34 µg Cu/ml). A new value of A_t' can be calculated using $R_a' = R_a(2.34)$

This process can be repeated until a constant value of A_t is achieved, as is shown in the following Table:

m = [Cu]	$R_a(m)$	$A_t(m)$	$m' = m \times A_t/100\%$
µg/ml	%	%	µg/ml
20.0	28	17	3.40
m'	$R_a' = R_a(m')$	$A_t' = (A_a(20)/R_a') \times 100\%$	
µg/ml	%	%	
3.40	41	11.7	
2.34	42	11.3	
2.26	43	11.2	
2.26	43	11.2	

The ultimate true absorption of 20 µg Cu/ml, corrected for the concentration dependence of R_a, is thus: A_t' = 11.2%, whereas the uncorrected value was: $A_t(20)$ = 17%.

Literature cited

1. Heth, D.A. & Hoekstra, W.G. (1965) Zinc-65 absorption and turnover in rats. I. A procedure to determine zinc-65 absorption and the antagonistic effect of calcium in a practical diet. J. Nutr. 85, 367-374.
2. Van Barneveld, A.A. & Van den Hamer, C.J.A. (1984) Influence of application of a purified diet on absorption of ^{65}Zn, ^{64}Cu, ^{54}Mn, ^{60}Co, ^{203}Pb, ^{115m}Cd and ^{75}Se, and particularly on metabolism of Zn in mice. To be published.
3. De Soete, D., Gijbels, R. & Hoste, J. (1972) In: Neutron activation analysis, pp. 505-506. Chemical Analysis, Vol. 34 (Ed. Elving, P.J. and Kolthoff, J.M.), Wiley-Interscience, London.
4. Larsson, B. & Ullberg, S. (1981) Whole-body autoradiography. J. Histochem. Cytochem. 29, 216-225.
5. Thomson, C.D. & Stewart, R.D.H. (1973) Metabolic studies of [^{75}Se]selenomethionine and [^{75}Se]selenite in the rat. Br. J. Nutr. 30, 139-147.
6. Burk, R.F., Brown, D.G., Seely, R.J. & Scaief III, C.C. (1972) Influence of dietary and injected selenium on whole-body retention, route of excretion, and tissue retention of $^{75}SeO_3^{2-}$ in the rat. J. Nutr. 102, 1049-1056.
7. Hansen, J.C. & Kristensen, P. (1979) The kinetics of ^{75}Se-selenium in relation to dose and mode of administration to mice. J. Nutr. 109, 1223-1233.
8. McConnell, K.P. & Portman, O.W. (1952) Excretion of dimethyl selenide by the rat. J. Biol. Chem. 195, 277-282.
9. Gitlin, D., Hughes, W.L. & Janeway, C.A. (1960) Absorption and excretion of copper in mice. Nature 188, 150-151.
10. Van Campen, D.R. (1971) Absorption of copper from the gastrointestinal tract. In: Intestinal absorption of metal ions, trace elements and radionuclides, pp. 211-227 (Skoryna, S.C. and Waldron-Edward, D., eds), Pergamon, Oxford, UK.
11. Davies, N.T. (1980) Studies on the absorption of zinc by rat intestine. Br. J. Nutr. 43, 189-203.
12. Smith, K.T. & Cousins, R.J. (1980) Quantitative aspects of zinc absorption by isolated, vascularly perfused rat intestine. J. Nutr. 110, 316-323.
13. Jackson, M.J., Jones, D.A. & Edwards, R.H.T. (1981) Zinc absorption in the rat. Br. J. Nutr. 46, 15-27.
14. Payton, K.B., Flanagan, P.R., Stinson, E.A., Chodirker, D.P., Chamberlain, M.J. & Valberg, L.S. (1982) Technique for determination of human zinc absorption from measurement of radioactivity in a fecal sample or the body. Gastroenterol. 83, 1264-1270.

15. Conrad, M.E. & Barton, J.C. (1978) Factors affecting the absorption and excretion of lead in the rat. Gastroenterol. 74, 731-740.
16. Mykkänen, H.M. & Wasserman, R.H. (1981) Gastrointestinal absorption of lead (^{203}Pb) in chicks: influence of lead, calcium, and age. J. Nutr. 111, 1757-1765.
17. Aungst, B.J. & Fung, H.-L. (1981) Kinetic characterization of in vitro lead transport across the rat small intestine. Mechanism of intestinal lead transport. Toxicol. Appl. Pharmacol. 61, 39-47.
18. Wapnir, R.A., Moak, S.A., Lifshitz, F. & Teichberg, S. (1979) Alterations of intestinal and renal functions in rats after intraperitoneal injections of lead acetate. J. Lab. Clin. Med. 94, 144-151.
19. Evans, G.W., Grace, C.I. & Votava, H.J. (1975) A proposed mechanism for zinc absorption in the rat. Am. J. Physiol. 228, 501-505.
20. Lönnerdal, B., Schneeman, B.O., Keen, C.L. & Hurley, L.S. (1980) Molecular distribution of zinc in biliary and pancreatic secretions. Biol. Tr. El. Res. 2, 149-158.
21. Evans, G.W., Johnson, E.C. & Johnson, P.E. (1979) Zinc absorption in the rat determined by radioisotope dilution. J. Nutr. 109, 1258-1264.
22. Flanagan, P.R., Haist, J. & Valberg, L.S. (1983) Zinc absorption, intraluminal zinc and intestinal metallothionein levels in zinc-deficient and zinc-replete rodents. J. Nutr. 113, 962-972.

4.6. PAPER V

INTESTINAL PASSAGE AND ABSORPTION OF SIMULTANEOUSLY ADMINISTERED ^{64}Cu AND ^{65}Zn AND THE EFFECT OF FEEDING IN MOUSE AND RAT.

A.A. Van Barneveld and C.J.A. Van den Hamer

ABSTRACT The intestinal passage and absorption of ^{64}Cu and ^{65}Zn, administered simultaneously by intragastric intubation, was investigated in mice and rats. In mice 50% of the intubated solution flowed directly into the small intestine. The clearance of Cu from the stomach was slow; at 2 hours post administration (p.a.) 22% of the dose of ^{64}Cu was still found in the stomach, as compared to 1% of the dose of ^{65}Zn. The intestinal passage of Cu was rapid and unabsorbed Cu was collected in the caecum, until defaecation occurred. Zn showed a remarkable retention in the small intestine; its accumulation in the caecum was not as strong as of Cu. Defaecation of unabsorbed Cu and Zn was highly stimulated by refeeding of the animals at 2 hours p.a. This refeeding also slightly reduced the absorption of Cu and Zn. A considerable quantity of tissue-bound ^{65}Zn was found in the small intestine at 24 hours p.a. both in starved mice (18%) and in refed mice (10%); the amount of tissue-bound ^{64}Cu in the small intestine was negligible (1.3% and 0.5%, resp.). In rats the absorption of ^{64}Cu seemed to occur only during the first 2 hours p.a., when most of the ^{64}Cu was still in the stomach. The intestinal passage of ^{64}Cu was slower and of ^{65}Zn more rapid than in mice. The ratio between the amount of ^{64}Cu and that of ^{65}Zn taken up in liver plus carcass at 24 hours p.a. was 0.19 in mice and 0.83 in rats.

Introduction

Intragastric intubation of an aqueous isotope solution is a much applied method of oral administration for the measurement of trace element absorption in laboratory animals. It guarantees well standardized administration conditions, in which contact and interaction between the trace element and food components can be avoided. It is also a clean method, because it limits the risks of external radioactive contamination as compared to isotope administration in drinking water or food. For these reasons the intragastric intubation, also called administration "by gavage", is often preferred above other methods of oral administration.

The absorption of a trace element from an intubated solution depends on the absorption capacity of the gastrointestinal tract and on the speed of intestinal passage. The absorption capacity of the intestine is influenced by conditions like developmental stage, state of health, trace element status of the body, and so on. The absorption of trace elements seems to be optimal in

the small intestine, whereas the stomach, the caecum and the colon probably have little or no absorption capacity for trace elements [1,2]. The small intestine seems to possess an absorption gradient over its full length [3] The speed of intestinal passage of a trace element depends on gastrointestinal motility, conditions of feeding after isotope intubation and the mode of interaction between the trace element and the gut wall.

This paper focuses on the intestinal passage of Cu and Zn in relation to some absorption characteristics, viz., the uptake in liver and carcass Cu and Zn were tested simultaneously to show the element specificity of the processes which determine intestinal passage and absorption. Also two feeding conditions, viz., continued starvation during 24 hours and refeeding 2 hours post administration, were tested. The experiments were carried out in mice using the radioactive isotopes ^{64}Cu and ^{65}Zn; an additional experiment with rats was done to show the animal specificity of the results.

Methods

Carrier-free ^{65}Zn was obtained from The Radiochemical Centre, Amersham, UK. ^{64}Cu (1 Ci/g) was produced by neutron irradiation of a Cu wire in the nuclear reactor of the Interuniversity Reactor Institute, Delft, The Netherlands. A purified diet on basis of casein (IRI-CB [4]) was obtained from Hope Farms BV, Woerden, The Netherlands.

Female Swiss Random mice, 4 weeks old, and male Wistar rats, 6 weeks old, were obtained from the Central Institute for the Breeding of Laboratory Animals-TNO, Austerlitz, The Netherlands. The animals were housed in macrolon cages with stainless steel lids and provided with glass drinking bottles with stainless steel drinking nipples During two weeks they were fed the purified diet ad libitum. After this adaptation period they were fastened during 20 hours prior to the experiments.

Experiment 1 To 5 groups of 4 mice 0.3 ml of an aqueous solution containing 1.5 µCi ^{64}Cu and 0.5 µCi ^{65}Zn (5 µg Cu/ml and 10 µg Zn/ml) was administered by intragastric intubation. At 5 times, viz., at once, 10 minutes post administration (p.a.) and 2, 6 and 24 hours p.a., one of the groups was sacrificed by bleeding. The gastrointestinal tract was isolated and divided into 8 segments, viz., stomach, duodenum, three equal parts of jejunum, ileum, caecum and colon (Fig 1) The segments of duodenum, jejunum and ileum were each rinsed with 5 ml of saline; the rinsing liquid containing the luminal contents was collected. The intestinal segments, the rinsing liquid, the liver and the remaining carcass were counted for their ^{64}Cu and ^{65}Zn content. During the experiment no food was provided.

Experiment 2 To 2 groups of 10 mice 0.3 ml of an aqueous solution containing 1.5 µCi ^{64}Cu and 0.5 µCi ^{65}Zn (5 µg Cu/ml and 10 µg Zn/ml) was administered by intragastric intubation. 10 Mice were fastened for another 24

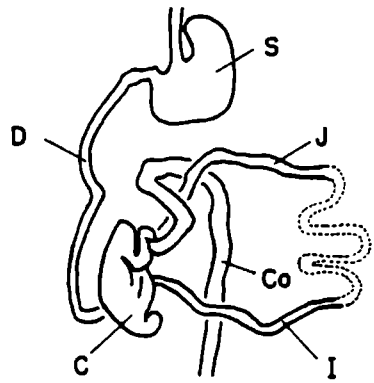


Fig. 1. Gastrointestinal segments used for the measurement of intestinal passage of ^{64}Cu and ^{65}Zn: stomach (S), duodenum (D), jejunum (J), ileum (I), caecum (C) and colon (Co). The division of the small intestine in duodenum, 3 parts of jejunum and ileum was arbitrary.

hours, the other 10 mice were fed the purified diet again at 2 hours p.a. From each group 5 mice were sacrificed by bleeding at 24 hours p.a. The segments of the gastrointestinal tract, the liver and the remaining carcass were collected and counted as described in experiment 1. The rinsing procedure was omitted. The remaining 5 mice in each group were fed the purified diet during the next four days. During this period the retention of ^{64}Cu and ^{65}Zn in these mice was measured by whole-body counting (^{64}Cu only during 1 day because of its short half-life of 12.8 hr). On the last day of counting the mice were sacrificed.

Experiment 3. To 5 rats 0.5 ml of an aqueous solution containing 2.5 µCi ^{64}Cu and 0.8 µCi ^{65}Zn (5 µg Cu/ml and 10 µg Zn/ml) was administered by intragastric intubation. At various times, viz. 10 minutes and 2, 6 and 24 hours p.a. under continued starvation, and 24 hours p.a. with refeeding at 2 hours p.a. one of the rats was sacrificed by bleeding. The segments of the gastrointestinal tract, the liver and the remaining carcass were collected as described in experiment 1. The rinsing procedure was omitted.

^{64}Cu and ^{65}Zn were measured simultaneously using an auto-gamma scintillation spectrometer (Packard 5120) for the organs and two small-animal whole-body counters for the carcass and for living animals. For mice a whole-body counter was used composed of two NaI crystals (3"x3") with wells (1.5"x2") positioned oppositely, thus forming the cavity in which the mouse was placed in a reproducible way (Fig. 2). Signals from both crystals were

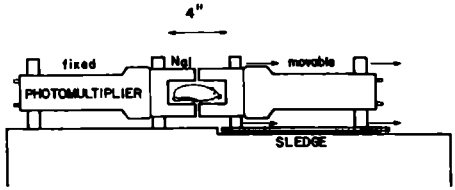


Fig. 2 Whole-body counter for mice. Two well-type NaI-crystals are positioned oppositely. The signals from both crystals are summed.

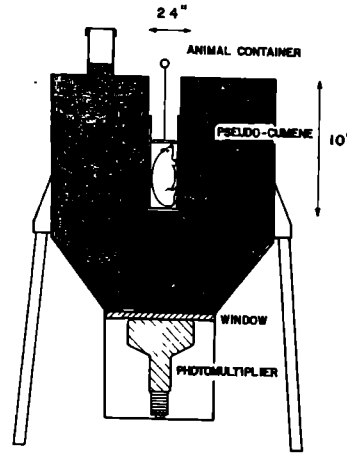


Fig. 3 Whole-body counter for rats. The well is positioned centrally in the cylindric metal container, which is filled with a liquid scintillator (pseudocumene).

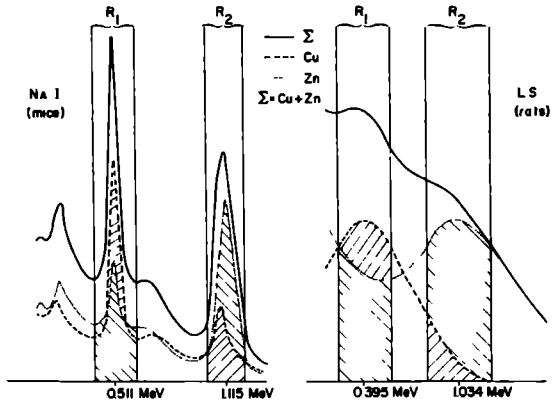


Fig. 4 ^{64}Cu and ^{65}Zn spectra obtained with the whole-body counter for mice (scintillation crystals: NaI) or that for rats (liquid scintilator: LS). The surface under the curve was measured in the regions R_1 and R_2 for ^{64}Cu and ^{65}Zn separately and for the sum-spectra (Σ). The main formulas used were:

$R_1(Zn) = f * R_2(Zn)$ $\qquad R_1(\Sigma) = R_1(Cu) + R_1(Zn)$

$R_2(Cu) = g * R_1(Cu)$ $\qquad R_2(\Sigma) = R_2(Zn) + R_2(Cu)$

The constants f and g can be calculated from the individual spectra of ^{65}Zn and ^{64}Cu.

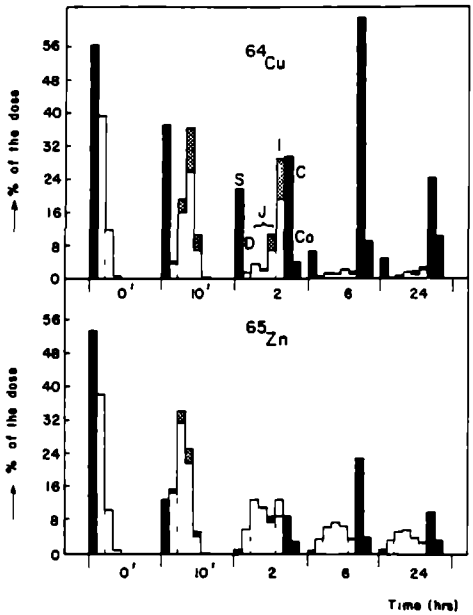


Fig. 5 ^{64}Cu and ^{65}Zn content of the gastrointestinal segments of mice at various times after intubation of the isotopes. S = stomach (black area); D, J and I = duodenum, jejunum and ileum (lightly shaded area = amount removable by rinsing); C and Co = caecum and colon (heavily shaded area).

summed. For rats a large scintillation liquid (pseudo-cumene) container fitted with a centrally positioned well (2.4"x10") was used (Fig. 3). All measurements were compared to a $^{64}Cu/^{65}Zn$ standard to eliminate differences in counting efficiency of the apparatus used. Calculation of the separate ^{64}Cu and ^{65}Zn activities was performed by correction of the measured activities for the mutual contribution of the isotopes (Fig. 4).

Results and discussion

Experiment 1. The passage of ^{64}Cu and ^{65}Zn through the gastrointestinal tract of mice during the first 24 hours p.a. (Fig. 5) showed a clear difference between Cu and Zn. Both rapidly entered the small intestine; the presence of significant and equal amounts of ^{64}Cu and ^{65}Zn in the duodenum and jejunum immediately after intragastic intubation indicates that part of the intubated solution (50%) flowed directly through the gastric pylorus into the

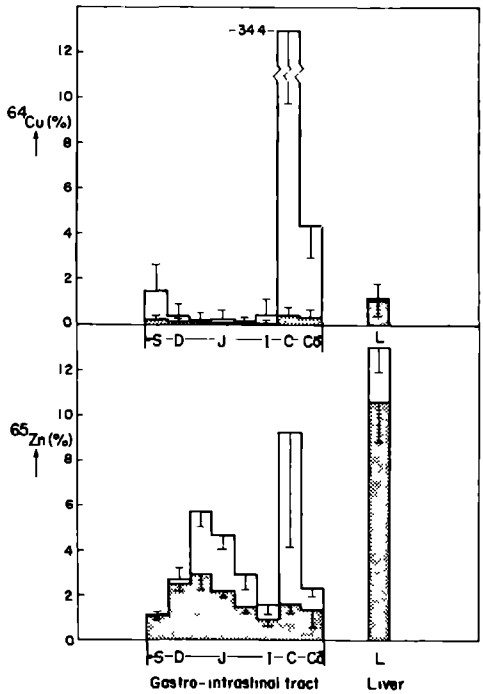


Fig. 6 Uptake of ^{64}Cu and ^{65}Zn in liver and carcass during 24 hours p.a. Each point represents the mean and standard deviation in 4 mice.

the small intestine. The clearance of ^{65}Zn from the stomach was rapid; at 2 hours p.a. only 1% of the dose was left. That of ^{64}Cu, however, was much slower; at 2 hours p.a. 22% was left in the stomach, probably bound to the epithelial layer.

In contrast to the Cu clearance from the stomach, that from the small intestine was very rapid. At 2 hours p.a. the bulk of ^{64}Cu had passed the jejunum and at 6 hours p.a. ^{64}Cu accumulated to a very high level in the caecum. The clearance of ^{65}Zn from the small intestine was much slower. 24 Hours p.a. significant amounts of ^{65}Zn were found in all parts of the small intestine. It is noteworthy that both ^{64}Cu and ^{65}Zn, during their passage through the small intestine, were almost completely bound to the intestinal wall. Only a small fraction could be flushed out with saline.

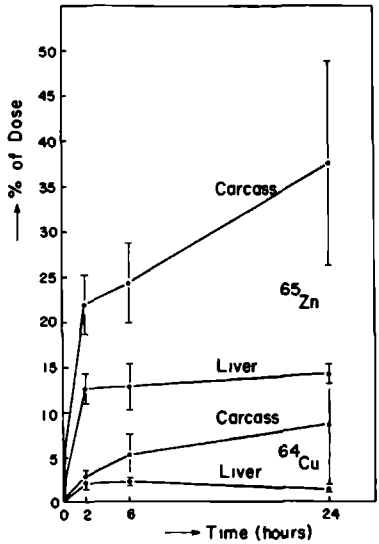


Fig. 7 ^{64}Cu and ^{65}Zn content at 24 hours p.a. in the gastrointestinal segments and in the liver (L) of mice starved continuously (area under the upper curve) or refed at 2 hours p.a. (shaded area). For explication of abbreviations: see Fig. 1.

The time-dependent uptake of ^{64}Cu and ^{65}Zn in the liver and carcass (Fig. 6) indicates that the absorption of Zn was much higher than that of Cu. The absorption mainly took place during the first 2 hours (34.6% for Zn, 5.0% for Cu). The contribution of the next 4 hours was relatively small for Zn (2.5% compared to 34.6%), but high for Cu (2.5% compared to 5.0%), probably because of the slow clearance of Cu from the stomach. The decrease in the ^{64}Cu content of the liver at 24 hours p.a. could be the result of excretion of ^{64}Cu in the bile [5]. There may also have been some incorporation of ^{64}Cu into caeruloplasmin, which is synthetized in the liver and secreted into the circulation [5].

Experiment 2 Fig. 7 shows the ^{64}Cu and ^{65}Zn content at 24 hours p.a. in the segments of the gastrointestinal tract and in the liver of starved mice and of mice refed at 2 hours p.a. The accumulation of ^{65}Zn in the caecum of the starved animals was less than one third of that of ^{64}Cu as a result of the higher retention (18%) of ^{65}Zn in the small intestine and of the higher absorption of ^{65}Zn. Refeeding at 2 hours p.a stimulated gastrointestinal emptying: the unabsorbed or endogenously excreted ^{64}Cu and ^{65}Zn, collected

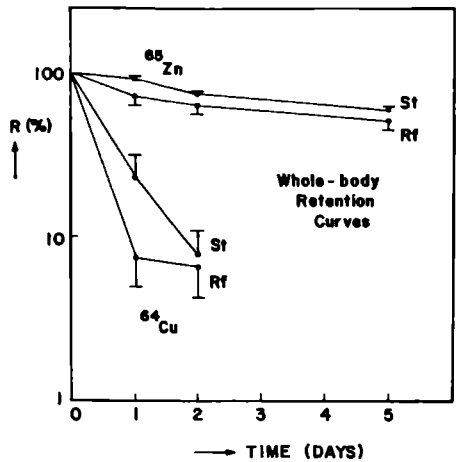


Fig. 8 Retention curves of ^{64}Cu and ^{65}Zn in mice starved during 24 hours p.a. (St) or refed at 2 hours p.a. (Rf). Each curve represents the mean and standard deviation in 5 mice. ^{64}Cu could not be measured for longer than 2 days due to its short half-life (12.8 hours).

in the caecum and colon, were almost completely removed by defaecation. A significant amount of ^{65}Zn (10%) remained, however, in the small intestine in spite of the passage of food. This ^{65}Zn could also originate from endogenous excretion or be bound to deeper intestinal layers.

Table 1 ^{64}Cu and ^{65}Zn content, expressed as % of the dose, in liver and carcass of fastened mice and mice refed at 2 hours p.a., measured at 24 hours p.a.

Mice	Liver		Carcass	
(n=5)	^{64}Cu	^{65}Zn	^{64}Cu	^{65}Zn
	%		%	
Starved	1.1±0.7	13±1	5.9±1.8	42±5
Refed	1.2±0.6	11±2 *	2.5±1.4 *	37±3 *

*) Significantly different by Student's t-test, $P < 0.05$.

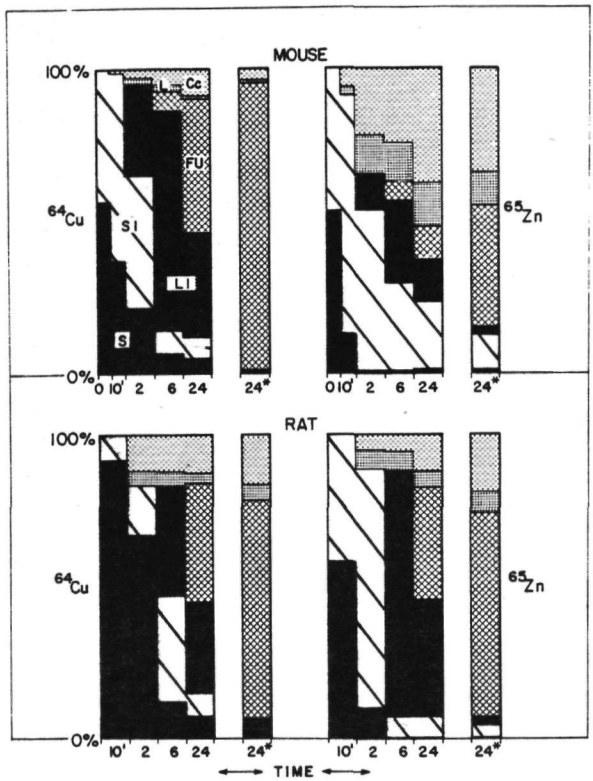


Fig. 9 Distribution of ^{64}Cu and ^{65}Zn in mice and in rats over stomach (S), small intestine (SI), large intestine (LI), faeces and urine (FU), liver (L) and carcass (Cc) at various times after intragastric intubation. The animals were starved continuously, or refed at 2 hours p.a. (*). Stomach and intestinal segments include luminal contents.

The defaecation effect was also seen in the retention curves of ^{64}Cu and ^{65}Zn in similarly treated mice, which were followed over a period of 5 days (Fig. 8). The difference in ^{64}Cu retention at 24 hours p.a. almost disappeared at 48 hours p.a. by defaecation of ^{64}Cu in the starved mice. The defaecation effect was less pronounced for ^{65}Zn due to its higher retention.

The data of ^{64}Cu and ^{65}Zn uptake in liver and carcass of starved or refed mice at 24 hours p.a. (Table 1) show that refeeding the mice at 2 hours p.a. reduced absorption of both ^{64}Cu and ^{65}Zn, as was already suggested by

the retention curves in Fig. 8. This should be taken into account, when feeding conditions are set up for trace element absorption experiments: food consumption shortly after oral trace element administration may influence the absorption of the element.

Experiment 3. Fig. 9 shows the distribution of ^{64}Cu and ^{65}Zn over the main metabolic compartments as found for rats; in the same figure these data are compared with those found for mice (a combination of the data presented in Fig. 5, 6 and 7). The common characteristics of mice and rats are the slow clearance of Cu from the stomach and the effect of refeeding at 2 hours p.a. on the defaecation of ^{64}Cu and ^{65}Zn. The rats showed a rapid uptake of ^{64}Cu in liver and carcass only during the first 2 hours p.a., when more than 67% of the dose was still in the stomach. This seems to suggest that rats may absorb more ^{64}Cu from the stomach than mice; however, it is also possible that between 2 and 6 hours p.a. excretion of absorbed ^{64}Cu in the bile of the rats compensated ^{64}Cu absorption. The absorption of ^{65}Zn from the small intestine seemed to be lower in rats than in mice. As a result the ratio between ^{64}Cu and ^{65}Zn uptake in liver plus carcass at 24 hours p.a. was much smaller in the mice (Cu/Zn=0,19) than in the rats (Cu/Zn=0,83). This result was obtained with only a few rats, so definite conclusions cannot be drawn.

In view of the simultaneous administration and measurement of ^{64}Cu and ^{65}Zn in the same animals the separation of both isotopes in the gut was not the result of an accidental difference in gastrointestinal motility, but of an element-specific physiological interaction with the intestinal wall. Almost all of the isotope content of the small intestine was bound to the intestinal wall and not to the luminal contents. Only a small fraction of this bound isotope seemed to be absorbed. Possibly the isotopes were bound to the glycocalyx [6] and to mucosal surface receptors or even absorbed into the mucosal cell, but later on released again into the lumen by abrasion of the glycocalyx, desquamation of the aged mucosal cell, return flux from the mucosal cell to the lumen [7] or exchange with stable metal from newly consumed food or from endogenous secretions. At longer time intervals endogenous excretion of previously absorbed isotopes in bile or pancreatic juice [5,8] may have contributed to the radioactivity in the intestinal segments. Uptake of absorbed ^{64}Cu and ^{65}Zn into the muscular and connective tissue of intestinal segments may also have contributed to their isotope content.

Acknowledgements

This work was supported by grant R712 from the Dutch Ministry of Public Health and Environmental Hygiene. The authors wish to thank Prof. J.P.W. Houtman and Dr. J.H.M. Van Tongeren for critical review of the manuscript. Mr. E.S. Oosterling is thanked for technical assistence.

References

1. Van Campen, D.R. & Mitchell, E.A. (1965) Absorption of Cu^{64}, Zn^{65}, Mo^{99}, and Fe^{59} from ligated segments of the rat gastrointestinal tract. J. Nutr. 86, 120-124.
2. Davies, N.T. (1980) Studies on the absorption of zinc by rat intestine. Br. J. Nutr. 43, 189-203.
3. Shubata, H. (1975) Absorption and excretion of zinc, cadmium and mercury in the gastrointestinal tract. Radioisotopes 24, 679-683
4. Van Barneveld, A.A. & Morse, H. (1982) A new diet for trace element research with rats and mice. Fed. Eur. Lab. Anim. Sci. Assoc., Düsseldorf, Z. Versuchstierk. 24, 24. IRI-Rapport 133-81-02, Delft, The Netherlands.
5. Mason, K.E. (1979) A conspectus of research on copper metabolism and requirements of man. J. Nutr. 109, 1979-2066.
6. Quarterman, J. (1982) A possible role for the glycocalyx in metal absorption. J. Physiol.-London 322, 23P.
7. Bonewitz, Jr, R.F., Foulkes, E.C., O'Flaherty, E.J. & Hertzberg, V.S. (1983) Kinetics of zinc absorption by the rat jejunum: effects of adrenalectomy and dexamethasone. Am. J. Physiol. 244, G314-G320.
8. Adler, M., Robberecht, P., Mestdagh, M., Cremer, M., Delcourt, A. & Christophe, J. (1980) The pancreatic secretion of zinc in man and rat. Gastroenterol. Clin. Biol. 4, 441-449.

47 PAPER VI

INFLUENCE OF MODE OF INJECTION - INTRAVENOUSLY OR INTRAPERITONEALLY - ON ORGAN DISTRIBUTION OF ^{65}ZN IN MICE

A.A. Van Barneveld, L. Prinsen and C.J.A. Van den Hamer

ABSTRACT A comparison was made between intravenous and intraperitoneal injections of ^{65}Zn at two dose levels (10 µg Zn and 0.3 µg Zn) with respect to the organ distribution of ^{65}Zn in mice. To the lower Zn dose histidine was added in a 10-fold molar ratio to Zn to minimize Zn losses by unintended local binding. The mice were sacrificed at five different times after injection and the organ distribution of ^{65}Zn was investigated both by whole-body autoradiography (dose: 10 µg Zn) and by dissecting and counting of the organs (dose: 0.3 µg Zn). Intraperitoneally injected ^{65}Zn was taken up from the peritoneal cavity within two hours and distributed over the body in a similar way as intravenously injected ^{65}Zn. However, at the lower dose at least 15% of i.p.-injected ^{65}Zn was retained in the peritoneal cavity, probably bound to covering linings of or diffused into abdominal organs (particularly the gastrointestinal tract). Beside this unintended local binding no difference in ^{65}Zn distribution could be observed between the two routes of administration.

Introduction

Parenteral administration of radioactive trace elements, in eliminating the step of absorption from the gut, can be helpful to the investigation of trace element metabolism. It is also a necessary tool to distinguish between apparent absorption and actual or true absorption, when a trace element is excreted from the body into the intestine, e.g., in the bile or digestive juice [1,2].

The most obvious route of parenteral administration of a trace element is its intravenous injection. Here the trace element enters the intravascular compartment at once. The disadvantages of this route are on the one hand the relative difficulty of intravenous injection in small laboratory animals (in mice injection into the tail vein requires some skill, particularly in case of coloured mice), and on the other hand the pulselike entrance of the trace element into the circulation, whereas absorption from the gut generally takes a few hours.

A convenient and gradual mode of parenteral administration is the intraperitoneal injection. This method requires little skill. The injected trace element is gradually taken up from the peritoneal cavity into the lymphatic system and the circulation. The speed of this process is better comparable to

that of absorption from the gut than intravenous injection.

In this report a comparison is made between the organ distributions of ^{65}Zn after intravenous and intraperitoneal injection in mice at two dose levels of Zn (10 µg and 0.3 µg). To the lower dose histidine was added in a 10-fold molar ratio to Zn to minimize Zn losses by local binding. The ^{65}Zn distributions were investigated at different times after injection both by whole-body autoradiography and by dissection and counting of organs.

Materials and methods

Female Swiss Random mice, 4 weeks old, were obtained from The Central Institute for the Breeding of Laboratory Animals - TNO, Austerlitz, The Netherlands. A purified diet (IRI-CB), based on casein as the protein source [3], was obtained from Hope Farms BV, Woerden, The Netherlands. The mice were fed the purified diet during 2 weeks. They were then fasted for 20 hours prior to ^{65}Zn injection. ^{65}Zn (2 Ci/g Zn) was obtained from New England Nuclear, Boston, Mass., USA.

Whole-body autoradiography. Five mice received an intraperitoneal (i.p.) injection and 5 mice an intravenous (i.v.) injection of 20 µCi ^{65}Zn in 0.25 ml acetate buffer (0.05 M, pH 5.6, 0.7% NaCl; 40 µg Zn/ml). Immediately after injection and at time intervals of 10 minutes and 2, 6 and 24 hours post injection (p.i.) one mouse of each group was sacrificed by CO_2-inhalation, deep frozen in n-hexane at approximately -80° and used for cryomicrotomy according to Larsson and Ullberg [4]. Whole-body sections of 50 µm thickness were made on adhesive tape using a whole-body cryomicrotome (PMV-200), freeze-dried and placed against Structurix D10 X-Ray film (Agfa-Gevaert). The exposure time was 65 hours.

Dissection and counting of organs. Forty mice received an i.p.-injection and 24 mice an i.v.-injection of 0.6 µCi ^{65}Zn in 0.3 ml acetate buffer (0.05 M, pH 5.6, 0.7% NaCl; 1 µg Zn/ml, histidine added in a 10-fold molar ratio to Zn). At time intervals of 10 minutes and 2, 6 and 24 hours p.i. 10 i.p.-injected and 6 i.v.-injected mice were sacrificed by bleeding. Several organs and tissues (liver, kidneys, pancreas, spleen, heart, complete gastrointestinal tract including mesentery, femure, remaining carcass) were collected and their ^{65}Zn content measured, using an auto-gamma scintillation spectrometer (Packard 5120).

Results and discussion

The whole-body autoradiograms of mice injected with ^{65}Zn at a dose level of 10 µg Zn are shown in Fig. 1 (intraperitoneal injection) and Fig. 2 (intravenous injection). Both a medial and a sagittal section was made. At t=0 the i.p.-injected ^{65}Zn was found diffusely spread over the peritoneal cavity; the i.v.-injected ^{65}Zn was found in the blood vessels. At 10 minutes post injection (p.i.) the i.p.-injected ^{65}Zn seemed to be bound to covering lin-

lngs of the abdominal organs, whereas the i.v.-injected ^{65}Zn was already taken up by pancreas, liver, duodenal wall and bone. At 2 hours p.i. the i.p.-injected ^{65}Zn appeared to be taken up from the peritoneal cavity and distributed over liver, pancreas, intestinal wall and bone in a similar way as the i.v.-injected ^{65}Zn; no essential difference could be observed anymore. Also at 6 and 24 hours p.i. both ^{65}Zn distributions appeared to be similar.

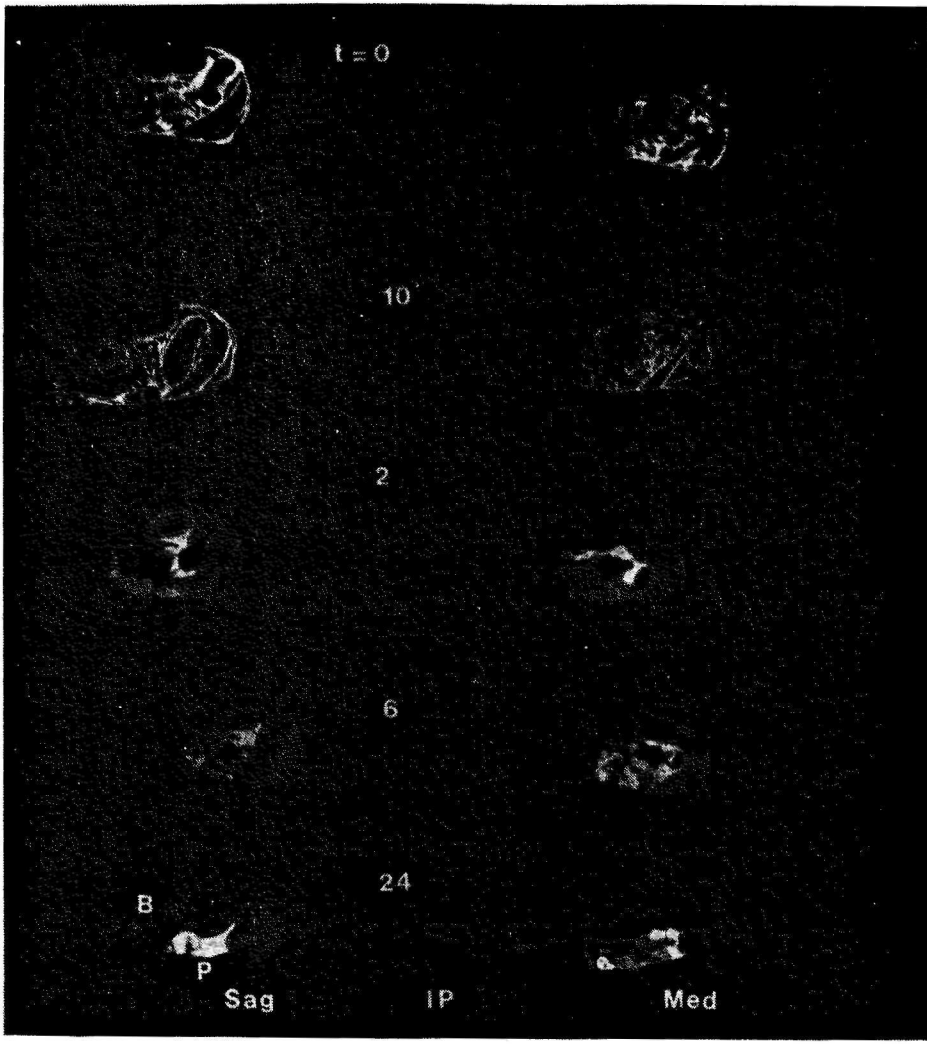
Remarkable in these autoradiograms is the rapid uptake of ^{65}Zn in bone: at 10 minutes p.i. already some ^{65}Zn was seen in the vertebrae. At 6 and 24 hours p.i. the concentration of both i.p.- and i.v.-injected ^{65}Zn was very high in the pancreas indicating the important role of endogenous excretion of ^{65}Zn in pancreatic juice. In the small intestine the ^{65}Zn activity was not found in the lumen, but in the epithelial layer (mucosa), suggesting an uptake of ^{65}Zn from the blood [5] or an absorption of excreted ^{65}Zn from the lumen by the mucosal cells. In one autoradiogram (i.v., 6 hours p.i.) ^{65}Zn could be seen in the lumen of the colon. The present autoradiograms showed good agreement with those made by Bergman and Söremark [6] of mice injected intraperitoneally with 500 µg Zn (50 µCi ^{65}Zn). In the latter autoradiograms, however, the injected ^{65}Zn seemed to be distributed more rapidly, which could be due to the higher amount of Zn injected.

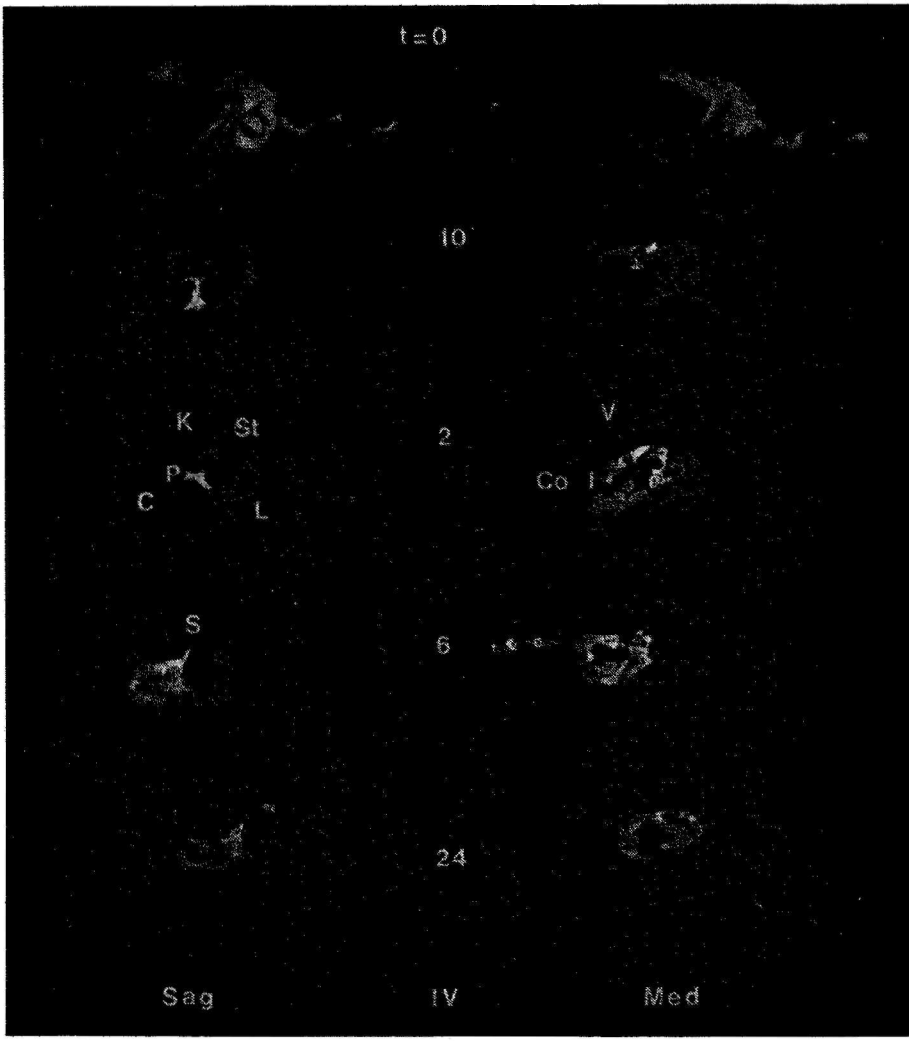
The ^{65}Zn contents of the organs of mice injected with a low dose of Zn (0.3 µg), which were determined by organ dissection and counting, are shown in Fig. 3. The rapid distribution of ^{65}Zn injected into the circulation and the slower uptake and distribution of ^{65}Zn injected into the peritoneal cavity are reflected by the maximum values of liver and kidney ^{65}Zn at 10 minutes p.i. in the i.v.-injected mice and at 2 hours p.i. in the i.p.-injected mice. At 2 hours p.i. the ^{65}Zn contents of all organs and tissues showed significant differences between the i.p.-injected and the i.v.-injected mice. After this time the form of the i.p.- and i.v.-curves was almost identical for all organs and tissues. This suggests that a redistribution of available ^{65}Zn took place independent of the mode of its administration. The ^{65}Zn content of

Legend of figures on page 100 and 101 :

Fig. 1 Autoradiograms of mice injected intraperitoneally with 20 µCi ^{65}Zn (10 µg Zn) and sacrificed at various times after injection (t=0, t=10', t=2, 6, 24 hr). Medial sections (showing the vertebrae) and sagittal sections (showing the left kidney) are presented on the right and left side, resp. L=liver; K=kidney; P=pancreas; S=spleen; St=stomach; I=intestine; C=caecum; Co=colon; B=bone; V=vertebrae.

Fig. 2 Autoradiograms of mice injected intravenously with 20 µCi ^{65}Zn (10 µg Zn) and sacrificed at various times after injection (see also Fig. 1).


t = 0
10
2
6
24
B
P
Sag
IP
Med


t=0
10
2
6
24
K
St
P
C
L
V
Co
S
Sag
IV
Med

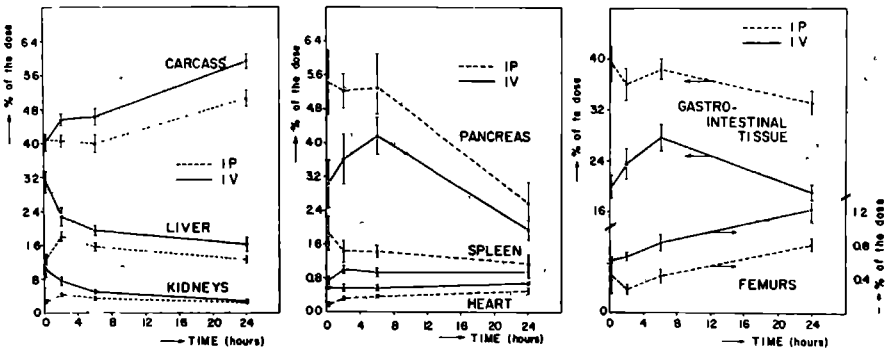


Fig. 3 Distribution of ^{65}Zn (0,3 µg Zn), injected intraperitoneally or intravenously in mice, at various times after injection. Data are presented as mean ± SD for 10 (i.p.) and 6 (i.v.) mice, resp.

liver, kidneys and pancreas decreased, whereas that of heart, bone and carcass increased, which is in agreement with earlier results [7]. The sharp decrease in the pancreas was probably due to endogenous excretion of ^{65}Zn in pancreatic juice [8]. The increase in the carcass could be related to increased ^{65}Zn uptake in muscle and in the bone, as is suggested by the increase of ^{65}Zn in the femurs. This process of redistribution may continue for several days [7,9].

In spite of this redistribution the differences between the ^{65}Zn contents of the organs and tissues of i.p.-injected and i.v.injected mice, observed at 2 hours p.i., remained unchanged. Table 1 shows that the negative differences in ^{65}Zn content of carcass, liver, kidneys, heart and femurs could be traced mainly to the binding of i.p.-injected ^{65}Zn to the gastrointestinal tract and to a small extent to the pancreas and spleen. It is plausible that at least 15% of the i.p.-injected ^{65}Zn was retained in the peritoneal cavity, bound to covering linings of or diffused into abdominal organs, and was unavailable for distribution over the body. Obviously the addition of histidine (in a 10-fold molar ratio to Zn) to the lower Zn dose to minimize Zn losses by local binding could not prevent some local binding of ^{65}Zn in the peritoneal cavity. This explanation is supported by the observation that the relative differences in the ^{65}Zn contents of carcass, liver, kidneys, heart and femurs between i.p.-injected and i.v.-injected mice were of a similar magnitude (Table 1). However, it is not excluded that also in the carcass, liver and kidneys of i.p.-injected mice some ^{65}Zn had not entered the tissue through the circulation, but through diffusion from the peritoneal cavity. Such local ^{65}Zn binding obviously is obscured by the much larger ^{65}Zn

Table 1 ^{65}Zn bound to organs and tissues of intraperitoneally (i.p.) or intravenously (i.v.) injected mice (dose: 0.3 µg Zn) at 24 hours after injection, expressed as % of the dose, and the difference between both (i.p.-i.v.), expressed as % of the dose (Δ) or as % of the organ content after i.v.-injection (Δ_{rel}=Δ/i.v.).

Tissue	i.p. (% of dose)	i.v. (% of dose)	Δ (% of dose)	Δ_{rel} (%)
Carcase	50.6	59.4	- 8.8	-15
Liver	12.5	16.1	- 3.6	-22
Kidneys	2.4	2.8	- 0.4	-15
Heart	0.5	0.7	- 0.2	-26
Femurs	0.8	1.2	- 0.4	-32
			——— +	
			-13.4	
G.I.-tract	33.1	19.1	+14.0	+73
Pancreas	2.0	2.6	+ 0.6	+24
Spleen	1.2	1.0	+ 0.2	+21
			——— +	
			+14.8	

binding from the circulation.

At higher doses as have been applied for the autoradiographic investigation (10 µg Zn) local binding of i.p.-injected Zn in the peritoneal cavity will also have occurred, probably in a quantitatively similar amount. On basis of the similar specific activities of the injected doses also the local binding of ^{65}Zn isotope should have been quantitatively similar. However, at the 0.3 µg dose this binding was about 15% of the ^{65}Zn activity, so at the 10 µg dose this should have been only 0.45% of the ^{65}Zn activity. Such a small fraction could not be observed in the autoradiograms, because it was obscured by the radioactivity of the ^{65}Zn bound after distribution through the circulation.

It is concluded that at higher doses (10 µg Zn) of injected ^{65}Zn there is no difference between the distributions of i.p.- and i.v.-injected ^{65}Zn when investigated after more than 2 hours after injection. At lower doses (0.3 µg Zn) the distribution of i.p.-injected ^{65}Zn during the first 24 hours after injection may partly be disturbed by local binding of ^{65}Zn in the peritoneal cavity.

Acknowledgements

Prof. J.P.W. Houtman and Dr. J.H.M. Van Tongeren are acknowledged for critical review of the manuscript.

References

1. Weigand, E. & Kirchgessner, M. (1976) Radioisotope dilution technique for determination of zinc absorption in vivo. Nutr. Metab. 20, 307-131.
2. Evans, G.W., Johnson, E.C. & Johnson, P.E. (1979) Zinc absorption in the rat determined by radioisotope dilution. J. Nutr. 109, 1258-1264.
3. Van Barneveld, A.A. & Morse, H. (1982) A new diet for trace element research with rats and mice. Z. Versuchstierk. 24, 24. IRI-Rapport 133-81-02, Delft, The Netherlands.
4. Larsson, B. & Ullberg, S. (1981) Whole-body autoradiography. J. Histochem. Cytochem. 29, 216-225.
5. Methfessel, A.H. & Spencer, H. (1973) Zinc metabolism in the rat. II. Secretion of zinc into intestine. J. Appl. Physiol. 34, 63-67.
6. Bergman, B. & Söremark, R. (1968) Autoradiographic studies on the distribution of zinc-65 in mice. J. Nutr. 94, 6-12.
7. Stortenbeek, A.J. & Van den Hamer, C.J.A. (1976) Binders of intravenously administered 65-zinc in rat liver cytoplasm. Bioinorg. Chem. 6, 313-327.
8. Adler, M., Robberecht, P., Mestdagh, M., Chremer, M., Delcourt, A. & Christophe, J. (1980) The pancreatic secretion of zinc in man and rat. Gastroenterol. Clin. Biol. 4, 441-449.
9. Jain, R.K., Gerlowski, L.E., Weissbrod, J.M., Wang, J. & Pierson, Jr., R.N. (1981) Kinetics of uptake, distribution, and excretion of zinc in rats. Ann. Biomed. Engin. 9, 347-361.

4.8 Summary

For the purpose of TE research with mice and rats, especially for the investigation of interactions between Ca and Mg and TE metabolism, a purified diet was developed characterized by an adequate but marginal mineral and TE content (IRI-CB purified diet) (4.2). On basis of adaptation experiments (4.3), particularly with Zn, it was decided that this diet should be fed at least two weeks before the start of metabolic experiments with radiotracered TE's. A larger adaptation period seemed undesirable, because young mice (at the end of their growing period) were preferred for this type of experiments.

With respect to Zn intragastric intubation and intraperitoneal injection appeared to be convenient modes of oral and parenteral administration of TE solutions (4.4). Absorption of Zn from an intubated solution was equal to that from drinking water, but much higher than that from food. This indicates that food and drinking water participate differently in TE absorption. Intraperitoneal injection appeared to be a convenient mode of parenteral administration of TE's necessary to determine the apparent retention of TE's. Fasting conditions before and after Zn administration appeared to have great impact on Zn absorption and retention. These conditions should therefore be rigorously standardized.

It was found that both apparent and true absorption and apparent retention of TE's (Cu, Zn, Pb, Se) may strongly depend on the TE concentration in the administered radioisotope solution (4.5). In this type of experiments one should be aware of isotope dilution effects caused by endogenous luminal TE pools, a recurrent but underestimated aspect in TE absorption studies. This aspect is discussed separately in Appendix II.

The processes of gastrointestinal passage and absorption showed essential differences between Cu and Zn and between mice and rats, indicating both element- and animal-specificity of such processes (4.6). The organ distribution of intraperitoneally injected Zn slightly differed from that of intravenously injected Zn during the first 24 hours after injection because of local binding of ip-injected Zn in the peritoneal cavity, particularly at lower doses (4.7).

The studies were performed to establish a set of standardized experimental conditions for TE metabolic research. These should rule out effects of unwillingly and unknowingly introduced variables and optimize sensitivity to effects of intentionally introduced variables. The results were used to set up an optimum experimental design for the investigation - by means of tracer studies - of the influence of Ca and Mg on TE absorption, the central subject of Chapter 5. The main features of this design are summarized in Section 4.9.

4.9 RECOMMENDATIONS FOR STUDIES OF TRACE ELEMENT METABOLISM WITH THE AID OF RADIOTRACERS IN MICE

1. A purified diet should be used containing adequate but marginal mineral and trace element concentration levels (e.g., IRI-CB purified diet).
2. Young (about 4 weeks old) female mice (e.g., Swiss Random) should be used. During at least two weeks prior to the metabolic experiment a purified diet should be given ad libitum in combination with demineralized drinking water.
3. Metabolic cages should be used to allow collection of faeces and urine and to minimize coprophagy. Galvanized materials should be avoided.
4. At least 10 mice per experimental group should be used for statistical evaluation.
5. The mice should be fasted during 20 hours before radioisotope administration and another 6 hours afterwards.
6. Oral administration (preferably intragastric intubation) should be combined with parenteral administration (preferably intraperitoneal injection) in another group of mice for evaluation of absorption and retention parameters.
7. Intragastric intubation of TE's in water should be combined with administration of TE's in food in another group of mice for evaluation of food effects.
8. The composition of the dose, particularly the concentration of the TE in the dose, should not be subject to occasional changes (e.g., by changes in the specific activity of radioisotope sources).
9. Retention curves should be measured during a period of at least 9 days (if this is allowed by the physical and biological half-life of the radioisotope).
10. Balance checks should be carried out by measuring faeces and urine.
11. Relevant organs should be collected and counted at the end of the experiment for additional information.
12. Attention should be given to the occurrence of isotope dilution effects.

CHAPTER 5

INFLUENCE OF CA AND MG ON TRACE ELEMENT METABOLISM

This chapter contains three studies on the influence of Ca and Mg on the absorption and retention of trace elements and an additional study on the influence of dietary Ca and Mg on mortality from Mg-deficiency.

5.1. Introduction.

The main topic of Chapter 5 is the investigation of the influence of Ca and Mg on the absorption and retention of trace elements (abbr. TE's). The method for measuring apparent absorption and apparent retention of radioactive TE's (an adapted version of the method of Heth and Hoekstra) has been described in Section 3.4 and 3.5. The experiments described in Section 5.2, 5.3 and 5.4 have been performed according to the recommendations mentioned in Section 4.9.

The effect of the presence of Ca and Mg on TE absorption has been investigated both in the aqueous system (by means of intragastric intubation of a radiotracered TE solution) and in the food system (by means of administration of portions of food mixed with radiotracered TE's). Ca and Mg (as $CaCl_2$ and $MgCl_2$, resp.) were added to the TE doses in concentrations (25 µmole/ml water or 25 µmole/g food), which were raised but not excessive in comparison with hard drinking water, and compared to not supplemented samples or samples supplemented with Na (as NaCl). Na supplementation was added to the set of experiments as a second blank to be able to distinguish possible effects of the anion (Cl^-) added together with the Ca or Mg. The food experiments were generally carried out using a purified diet containing no free Ca- or Mg-salts, to prevent obscuring of effects by such food minerals or by unrefined food components (fiber, phytate). An adequate purified diet (containing 100 µmoles of free Ca and 20 µmoles of free Mg per g as $CaCO_3$ and $MgSO_4.7H_2O$) was also incorporated into the series of foods to be tested.

Radiotracered TE's have been injected as well, viz. intraperitoneally in combination with addition of Ca or Mg to the drinking water. This part of the experiments should particularly allow the observation of an effect of water hardness on the excretion of TE's.

Ca and Mg could influence TE absorption not only by their presence, but also indirectly through the Ca- or Mg-status of the organism. To investigate such indirect effects experiments have been carried out in which mice were fed Ca- or Mg- deplete diets (diets without free Ca- or Mg-salts) or Ca- or Mg-replete diets (diets with super-adequate additions of Ca- or Mg-salts) during two or more weeks prior to TE administration. This period was considered long enough to reduce or improve the Ca- or Mg-status. The described

experiments have been carried out with the seven TE's selected and are reported in Section 5.2 (Zn, Cu, Co, Se, Cd), 5.3 (Mn) and 5.4 (Pb and Cd).

The experiments with Ca- or Mg-deplete diets led to observations on mortality from Mg-deficiency, which are presented in Section 5.2 and 5.5. Section 5.5 in particular describes the effects of various dietary Ca- and Mg-intakes on mortality and on the Ca- and Mg-content of the heart muscle.

5.2. PAPER VII *

INFLUENCE OF CA AND MG ON METABOLISM OF ZN, CU, CO, CD AND SE IN MICE

A.A. Van Bameveld, C.J.A. Van den Hamer and J.P.W. Houtman

ABSTRACT In view of the inverse correlation between drinking water hardness and cardiovascular diseases observed in epidemiological surveys in different countries, the effect of Ca and Mg on the metabolism of Cd, Co, Cu, Zn and Se (as selenite) was investigated in mice. Attention was focused on the absorption of these trace elements when given in a solution by stomach tube or mixed with food, and on their retention after intraperitoneal administration. The apparent absorption of Cd, Co, Cu and Zn was reduced and of Se increased by mixing with food in comparison with gastric intubation. The effects of Ca and Mg, added in raised but non-excessive amounts as compared to drinking water levels, were generally small. Reduction of absorption was found for Co (intubated) and Cd (mixed with food) by both Ca and Mg and for Zn (intubated) by Ca. For Co also a reduction of absorption was found in case of mice fed a Ca- and/or Mg-poor diet. Mice fed a Mg-poor diet died after several weeks. This effect was not observed in mice fed the same diet also poor in Ca. Calcium therefore appears to play a significant role in the mortality caused by Mg deficiency.

Introduction

The frequency of death from cardiovascular diseases is often reported to be negatively correlated with the hardness of drinking water [1]. Therefore, the question arises whether softening of drinking water is advisable. This in turn raises the question how the components of water hardness influence our health. One hypothesis states that Ca and Mg influence the absorption of trace elements or their metabolism in general. The purpose of this work was to test this hypothesis for several trace elements, both essential and toxic: Cd, Co, Cu, Zn and Se. The interest in Cu and Zn arises from the suggested hypertensive effect of Cu deficiency in relation to the Zn status [2]. Selenium deficiency was found to be related to Keshan disease, a cardiomyopathy observed in the Keshan region in China [3]. Cadmium has been suggested to raise blood pressure after long-term low-level exposure [4]. Cobalt has been found to cause cardiomyopathy in heavy beer drinkers consuming Co-containing

* This Paper has been presented on the 16th Annual Conference on Trace Substances in Environmental Health, Columbia, Missouri, May-June 1982. Because it was prepared earlier than Papers VIII and IX, minor differences may exist in formulation and terminology.

beer [5]. The role of trace elements in drinking water in the development of cardiovascular diseases recently has been discussed, leaving many questions to be answered [6]. This paper treats some aspects of this problem.

Materials and methods **

Female Swiss Random mice, 3-4 weeks old, were obtained from the Central Institute for the Breeding of Laboratory Animals - TNO, Austerlitz, The Netherlands. IRI-CB diet, a purified diet containing low but adequate levels of minerals and TE's [7] (see Table 1), and its Ca- and Mg-poor variant, the IRI-CB/P diet, were obtained from Hope Farms, Woerden, The Netherlands. Ca-,Mg-poor IRI-CB/P purified diet has the same composition as IRI-CB purified diet, except that Ca and Mg salts are omitted.

Table 1. Composition of IRI-CB purified diet.

Ingredient	%
Glucose	50.45
Corn starch	15.0
Casein	20.0
Sun flower seed oil	4.0
Fiber (α-cellulose)	5.0
dl Methionine	0.2
Choline chloride	0.3
Minerals [1]	3.95
Trace element mix (NETEM-80) [2]	0.1
Vitamin mix [3]	1.0

[1] Minerals (%) : $NaH_2PO_4.2H_2O$, 1.5; KCl, 0.7; $CaCO_3$, 1.0; $MgSO_4.7H_2O$, 0.5; $Na_2SiO_3.9H_2O$, 0.25. [2] Composition in mg/kg diet : Fe_2O_3, 85.9; $MnSO_4.H_2O$, 153.8; ZnO, 25.6; $CuCO_3.Cu(OH)_2$, 17.4; $NiCl_2.6H_2O$, 10.12; NaF, 5.52; $CrCl_3.6H_2O$, 10.25; $SnCl_2.2H_2O$, 3.80; KIO_3, 0.506; $Na_2MoO_4.2H_2O$, 0.504; Na_2SeO_3, 0.329; NH_4VO_3, 0.230; $Na_2HAsO_4.7H_2O$, 0.477; $CoSO_4.7H_2O$, 0.416; $Na_2B_4O_7.10H_2O$, 0.882; glucose to make 1000 mg/kg diet [3] Composition in IU or mg/kg diet : Retinyl acetate, 16.000 IU; cholecalciferol, 1.400 IU; Menadione, 12; phylloquinone, 2; dl α-tocopheryl acetate, 85; thiamine HCl, 20; riboflavine, 12; pyridoxine HCl, 15; nicotinic acid, 40; dl calcium pantothenate, 35; vitamin B 12, 0.05; d biotin, 0.4; folic acid, 8; myo-inositol, 500 mg.

Isotopes were obtained from New England Nuclear, Boston, USA (^{60}Co; ^{115m}Cd; ^{75}Se as selenite) and The Radiochemical Centre, Amersham, UK (^{65}Zn). ^{64}Cu was prepared by neutron activation in our institute.

**) Abbreviations and symbols used (in text and figures):
TE's = trace elements; i.g. = intragastrically; i.f. = in food (IRI-CB diet); i.f.* = in food (IRI-CB/P diet); i.p. = intraperitoneally.
-, Na, Ca, Mg = blank, Na-, Ca-, Mg-addition, resp.
A_a = apparent absorption; R_a = apparent retention; A_n and R_n = retention on day n of TE's administered orally and parenterally, resp.
F = faecal excretion; U = urinary excretion.
* = significantly different ($P<0.05$) from blank.
** = significantly different ($P<0.05$) from both blank and Na-addition.

The following experiments were carried out:

Experiment 1. Swiss mice were fed the IRI-CB diet during a period of 2 weeks before the start of the experiment. Food and demineralized drinking water were given ad libitum. After this adaptation period the animals were housed in metabolic cages and fasted for 24 hr. Then radioactively labeled TE's were administered as follows:

1a. I.g.-administration. In 5 x 4 groups of 10 mice ^{115m}Cd, ^{60}Co, ^{65}Zn, ^{75}Se (0.3 µg) or ^{64}Cu (1.5 µg) were intubated into the stomach as a solution in water as such or supplemented with NaCl, $CaCl_2$ or $MgCl_2$ (50, 25, 25 µmole/ml, resp.). Intubation volume was 0.3 ml.

1b. I.f.-administration. To 5 x 5 groups of 10-12 mice ^{115m}Cd, ^{60}Co, ^{65}Zn, ^{75}Se (2.0 µg per group) or ^{64}Cu (10.0 µg per group) were administered as a mixture with 10 g of food, viz., IRI-CB/P diet as such or supplemented with NaCl, $CaCl_2$ or $MgCl_2$ (50, 25, 25 µmole/g, resp.) (i.f.^{m}), and IRI-CB diet as such (i.f.). The food mixture was made available to the groups for 1.5 hr. The consumption per mouse showed a maximum relative standard deviation of 39%. In case of dose-size dependence the absorption per mouse was recalculated for the average consumption.

1c. I.p.-administration. In 4 x 4 groups of 10 mice ^{60}Co, ^{65}Zn, ^{75}Se (0.3 µg) or ^{64}Cu (1.5 µg) were injected intraperitoneally as a solution in acetate buffer (0.05 M; pH 5.6; 0.7% NaCl). Injection volume was 0.3 ml. Demineralized water was given ad libitum as such or supplemented with NaCl, $CaCl_2$ or $MgCl_2$ (50, 25, 25 µmole/ml, resp.).

In all experiments NaCl additior was twice molar as compared with $CaCl_2$ and $MgCl_2$ and served as a second control.

Experiment 2. During 5 weeks, 8 groups of 11 mice were fed diets with different Ca and Mg contents (Table 2). In all mice ^{64}Cu (1.5 µg), ^{75}Se, ^{60}Co and ^{65}Zn (0.3 µg) were intubated into the stomach as a solution in water : ^{64}Cu during the 3rd week, ^{75}Se during the 4th week, ^{60}Co and ^{65}Zn together during the 5th week. Intubation volume was 0.3 ml. At time of ^{75}Se counting ^{64}Cu had decayed to an undetectable level. ^{75}Se, being low-energetic, did not interfere with the $^{65}Zn/^{60}Co$ counting. ^{65}Zn and ^{60}Co measurements were corrected for their mutual interference.

In all experiments 6 hours after TE administration food was given again ad libitum. Retention of TE's was measured by whole-body counting during 9-12 days, starting immediately after administration. As a counter a container was used filled with pseudocumene as a scintillator and equipped with a centrally positioned well (2"x10") for insertion of the mouse. A second system with a higher resolution was used for whole-body counting of a mixture of isotopes. This system consists of 2 well (1.5"x2") type NaI crystals (3"x3"), positioned oppositely, thus forming the cavity in which the mouse can be kept during counting. The signals coming from both crystals are summed.

Table 2 Composition of steady diets A-G.

Nr.	Diet	Addition Ca (µmole/g)	Addition Mg (µmole/g)	Type
A	IRI-CB/P	—	—	Ca-,Mg-poor
B	IRI-CB/P	100	—	Mg-poor
C	IRI-CB/P	—	20	Ca-poor
D	IRI-CB/P	100	20	Standard
D'	IRI-CB	—	—	Standard
E	IRI-CB	250	—	Ca-rich
F	IRI-CB	—	50	Mg-rich
G	IRI-CB	250	50	Ca-,Mg-rich

Diet D and D' are equivalent.

Apparent absorption A_a of orally administered TE's and apparent retention R_a of parenterally injected TE's were calculated by extrapolation of the linear part of the semi-logarithmic retention curve to time t=0 (Fig. 1). In case of the short-living isotope ^{64}Cu ($t_{1/2}$=12.8 hr) A_a and R_a were replaced by the retention measured on day 3 (A_3 and R_3, resp.). Faeces and urine were collected and measured separately.

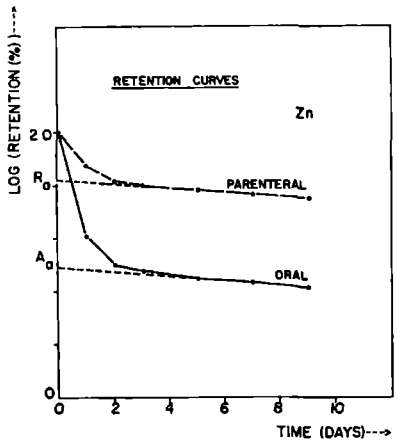


Fig. 1 Two arbitrary examples of retention curves used for determination of apparent absorption (A_a) and apparent retention (R_a), resp.

Results

1a,b. Influence of Ca and Mg in the dose on absorption of orally (i.g./i.f.^{m}/i.f.) administered TE's.

Fig. 2 shows A_a of Cd, Co, Zn and Se, and A_3 of Cu, after i.g./i.f.^{m}/i.f.-administration. Absorption of the cationic TE's was generally reduced by mixing with food. Apparent absorption of the anionic Se was increased by mixing with food. Cadmium and Cu were absorbed less when mixed with standard diet than when mixed with the Ca-,Mg-poor diet as such. Apart from differences as a result of the mode of administration significant effects of Ca and/or Mg addition in comparison with both controls were found for i.f.^{m} Cd (Ca, Mg), i.g.-Co (Ca, Mg) and i.g.-Zn (Ca).

1c. Influence of Ca and Mg in drinking water on retention of parenterally (i.p.) administered TE's.

Fig. 3 shows R_a of Co, Zn and Se and R_3 of Cu after i.p.-administration. No significant differences were observed between mice consuming Ca- or Mg-containing drinking water and controls consuming demineralized or Na-containing water.

2. Influence of Ca and Mg levels in the steady diet on incidental TE absorption (i.g.-administered).

Fig. 4 shows A_a of Co, Zn and Se, and A_3 of Cu, after i.g.-administration in mice fed diets with various Ca and Mg levels (Table 2) during several weeks. Diet D and standard diet D' are equivalent and may be considered as mutual controls. Much Ca in the diet (E, G) tends to reduce Zn absorption; however, the differences found were not significant. The high Zn absorption in case of diet D was not found in case of diet D, this result is not understood. Apparent absorption of Se was increased in case of the Ca-rich diet E. Livers of mice fed the Ca- and Mg-rich diets E, F, G were found to contain raised ^{75}Se levels. In case of the Ca- and Mg-poor diets A, B, C Co absorption was reduced.

Eight out of 11 mice in group B died in the period between 2 and 5 weeks, apparently when being frightened during handling. The acute death was accompanied by convulsions and squeaking. In the other groups no deaths occurred. This result was reproduced in another experiment with 4 groups of 10 mice using diets A, B, C, D. After 26 days, when 5 mice out of 10 in group B had died, all remaining mice were anaesthetized (CO_2 gas) and their blood sampled via the plexus orbitalis. Calcium and Mg were determined in the plasma by automated titration. The Ca/Mg ratio was raised in both Mg-poor groups (A and B) as compared with the standard group D (Table 3).

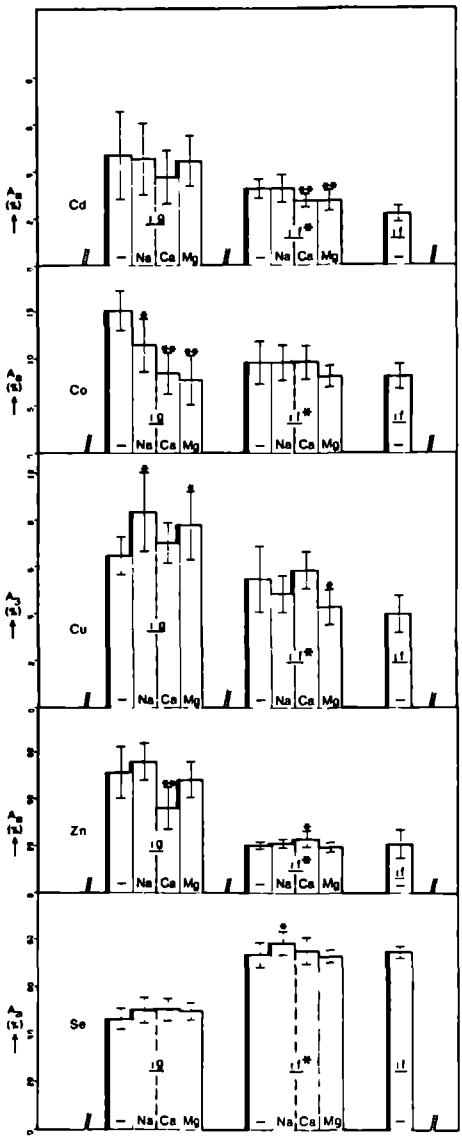


Fig. 2 Apparent absorption data (A_a including s.d.) for Cd, Co, Cu, Zn and Se as influenced by various minerals in the dose (see exp. 1a,b).

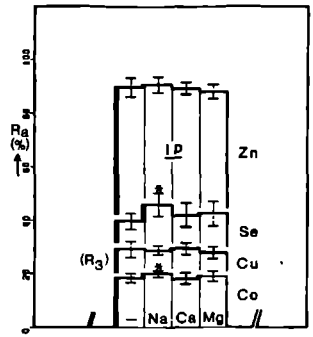


Fig. 3 Apparent retention data (R_a including s.d.) for Co, Cu, Zn and Se as influenced by various minerals in the drinking water (see exp. 1c).

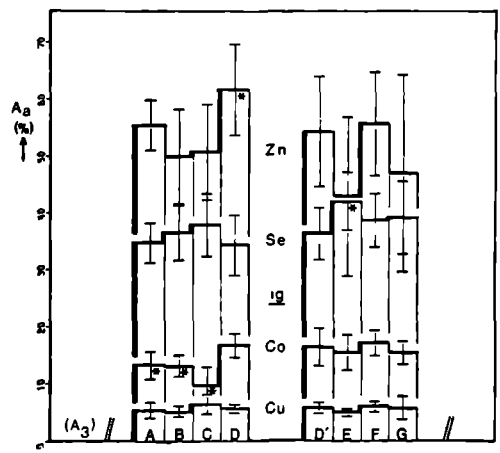


Fig. 4 Apparent absorption data (A_a including s.d.) for Co, Cu, Zn and Se as influenced by Ca and Mg levels in the steady diet (see exp. 2).

Table 3 Ca and Mg concentrations and Ca/Mg ratio in plasma of mice fed diets A, B, C, D.

Diet	Type	Ca (µmole/ml)	Mg (µmole/ml)	Ca/Mg ratio
A	Ca-,Mg-poor	0.34±0.06*	0.11±0.02	3.4±0.9*
B	Mg-poor	0.29±0.03	0.09±0.02*	3.5±0.8*
C	Ca-poor	0.23±0.02*	0.10±0.01*	2.3±0.3
D	Standard	0.26±0 03	0.13±0.02	2.1±0.2

*) Significantly different ($P<0.02$) from standard.

Discussion

TE administration i.g. (to a fasting animal) and if represent 2 extreme conditions in TE intake, viz., no and full contact between TE's and food components. The contribution of both extremes will depend strongly on consumption pattern, both in mice and in man : drinking between meals corresponds more to intragastric intubation, while drinking during meals corresponds more to mixing with food. In normal living practice the consumption pattern will vary.

Intubation of a TE solution is in fact a model for drinking water consumption on an empty stomach with the advantage of constant and known dosage (elimination of the possible influence of dose-size on absorption) and short time of administration (no risk of excretion before counting). It is specially suited for the measurement of direct interactions on the absorption level between minerals and TE's. When given on an empty stomach the intubated solution flows into the gastrointestinal tract and gets into direct contact with intestinal villi. In this way competition between minerals and TE's for mucosal transport systems may have an optimal chance to occur.

On the other hand food components may play an essential role in the interaction between minerals and TE's. Calcium or Mg may influence TE interaction with binding sites on the food components, thus changing availability for absorption. To optimize effects of mineral additions to the food the experiments were preferentially carried out with the Ca-,Mg-poor IRI-CB/P diet. This diet does not contain free Ca and Mg salts and is therefore more comparable to natural foods, in which Ca and Mg will be present in a bound form.

Except for Se, TE's mixed with food had a lower A_a than TE's given by stomach tube. Binding of TE's to food components may be responsible for this effect. In case of Cu and Zn, dilution of radioactive tracer with "cold" Cu and Zn from the food may also have reduced A_a. Mixing with standard diet reduced the absorption of Cd and Co as compared with the Ca-,Mg poor diet as

such, suggesting an influence of the free minerals in the standard diet. Apparent absorption of Se was higher when mixed with food than when given by stomach tube. As Se was given as the anionic selenite, its interaction with food components and mucosa can be expected to be different from those of the cationic TE's. Probably the uptake of selenite from food, which is more gradual, promotes metabolic conversion and incorporation, whereas the uptake from an intubated solution, which is more rapid, promotes urinary excretion of Se. From the balance of whole-body retention, urinary excretion and faecal excretion of ^{75}Se over the first 3 days after administration (Fig. 5) we may conclude that the true absorption of selenite under all conditions is very high and that indeed urinary excretion is higher in case of intragastric intubation.

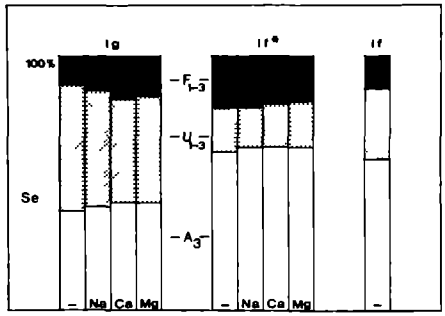


Fig. 5 Balance of Se over the first 3 days as influenced by various minerals in the dose (supplement to Fig. 2).

When the influence of Ca and Mg in the absorption experiments is considered, it must be kept in mind that the quantities of Ca and Mg added to food and to intubated solutions were about 5 times as high as the quantities of Ca and Mg which may be present in extremely hard drinking waters. Nevertheless most effects found were relatively small (exp. 1a,b; Fig. 2). The strongest effects were found for Co. Considering the Co balance over the first 2 days (Fig. 6) one sees that the faecal excretion of i.g.-administered Co is increased by Ca and Mg in comparison with controls, suggesting a reduced true absorption. A large part of the absorbed Co is rapidly excreted in the urine. The lower urinary excretion of Co in case of Ca and Mg addition makes the ultimate differences in retention less pronounced.

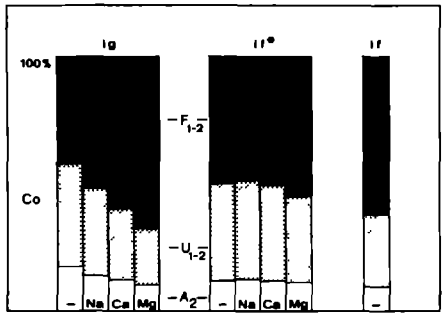


Fig. 6 Balance of Co over the first 2 days as influenced by various minerals in the dose (supplement to Fig. 2).

The absorption of Cd was decreased by Ca and Mg in presence of food. Although the effect was small, it may be important, because Cd is strongly accumulated in the body (kidney). A small difference in absorption eventually may cause a considerable difference in body burden, which might be a potential hazard in a large population.

No influence of raised Ca and Mg levels in drinking water on the endogenous excretion of TE's could be demonstrated (exp. 1c; Fig. 3). The effects of very low or raised Ca and Mg levels in the steady diet on incidental TE absorption (i.g.) were also only marginal (exp. 2; Fig. 4), taken into account that the conditions chosen were rather extreme. In fact they were insignificant in comparison with the mortality found in mice fed the Mg-poor diet B. The presence of Ca appears to play a significant role, as the mice fed the Ca-,Mg-poor diet A were not affected. Probably Ca binds to sites originally occupied by Mg, thus disturbing metabolic processes. The acute death of the mice fed the Mg-poor diet therefore might be the result of a disturbed heart function, caused by such a Ca/Mg exchange. Indeed it is known that Mg causes dysfunction of the heart [8]. We believe that our results contribute to the hypothesis that Mg could be the "water factor" in hard drinking water, which protects against cardiovascular disease [1]. However, if a diet low in Mg is a risk factor, then the Ca/Mg ratio in the total diet (including drinking water) may be important.

Conclusion

Although under the experimental conditions tested some effects of Ca and Mg were observed, there was no evidence that realistic Ca and Mg levels in drinking water will influence the metabolism of Cu, Zn and Se. Our results with Co and Cd require further investigation. Some results point to Mg as the "water factor" and furthermore indicate that the Ca/Mg ratio in food and water may be important.

Acknowledgements

Ms. L.M. Van Westing, Ms. H.K. Terlouw and Mr. E.S. Oosterling are thanked for technical assistence.

Literature cited

1. Neri, L.C. & Johansen, H.L. (1978) Water hardness and cardiovascular mortality. Ann. N.Y. Acad. Sci. 304, 203-219.
2. Klevay, L.M. (1975) Coronary heart disease, the zinc-copper hypothesis. Am. J Clin. Nutr. 28, 764-774.
3. Anonymous (1980) Prevention of Keshan cardiomyopathy by sodium selenite. Nutr. Rev. 38, 278-279.
4. Schroeder, H.A. (1965) Cadmium as a factor in hypertension. J. Chron. Dis. 18, 647-656.
5. Alexander, C.S (1969) Cobalt and the heart. Ann. Int. Med. 70, 411-413.
6. Proc. Int Conf. Drinking water and cardiovascular disease (1980) J. Environ. Pathol Toxicol. 4, 1-326.
7. Van Barneveld, A.A. & Morse, H. (1981) A new diet for trace element research with rats and mice Presented at 1st Sci. Meet. Fed. Eur. Lab. Anim. Sci. Assoc., Düsseldorf, BRD.
8. Iseri, L.T., Freed, J. & Bures, A.R. (1975) Magnesium deficiency and cardiac disorders Am. J Med. 58, 837-846

5.3. PAPER VIII

INFLUENCE OF CA AND MG ON METABOLISM OF MN IN MICE

A.A. Van Barneveld and C.J.A. Van den Hamer

ABSTRACT The influence of high but not extreme concentrations of Ca and Mg in water (25 µmole/ml) and of similar concentrations in food (25 µmole/g) on the absorption and retention of ^{54}Mn in mice was investigated. Addition of either Ca or Mg reduced the absorption of ^{54}Mn from water (administered by intragastric intubation) and from food (a portion made available for consumption during 15 hours) by more than 50%. The retention of intraperitoneally injected ^{54}Mn was not influenced by addition of Ca or Mg (25 µmole/ml) to the drinking water. In mice fed a Ca-deplete diet during two weeks prior to dosage, the absorption of ^{54}Mn from an intubated aqueous solution was increased by more than 100% compared to that in mice fed a Ca-adequate diet. In mice fed during two weeks a Mg-deplete diet or a Ca- or Mg-replete diet, no changes in the absorption of ^{54}Mn were observed. In mice fed a Ca,Mg-deplete diet after ^{54}Mn administration, the turnover of absorbed ^{54}Mn was strongly increased probably due to increased absorption of stable Mn from the diet. The results indicate that the uptake of Mn could be higher in soft water regions than in hard water regions

Introduction

The normal daily intake of manganese in man does not seem to be a point of much concern to nutritionists and toxicologists. On the one hand, the daily intake of Mn seems generally to be sufficient to cover the estimated requirements and dietary deficiencies of Mn are unknown [1]. On the other hand, Mn intoxications have been reported only in Mn miners [2]. Marienfeld and Collins [3] reviewed the role of Mn in the biological system and concluded that an increased uptake of Mn could have detrimental effects on human health with respect to cardiovascular diseases, cancer, and other diseases. Marienfeld also suggested that drinking water hardness, notably Ca, could influence the uptake of Mn from drinking water (personal communication).

Several animal studies indicate that Mn uptake from the food may be influenced by Ca in the drinking water or the food. Schroeder et al [4] have found that in rats given soft drinking water during more than a year the mean Mn content of the liver was 64% of that in rats given hard drinking water, although the difference was not significant. In contrast, Ingols and Craft [5] have found a three-fold increase of the Mn content in the liver of mice given soft drinking water during a period of three weeks compared to mice given hard drinking water. Lassiter et al [6] and Pond et al [7] found a

significant increase of the Mn content in the liver of rats fed a low Ca diet. In experiments with ^{54}Mn Lassiter et al. [6] also found an increased turnover of oral and parenteral doses of ^{54}Mn in such rats. On the ground of these indications it was considered worthwile to investigate the influence of Ca and Mg, the main components of drinking water hardness, on the metabolism of Mn.

The present study reports effects of both Ca and Mg (as $CaCl_2$ and $MgCl_2$) on the absorption in mice of Mn from water and from food. Effects of Ca- or Mg-depletion and of Ca- or Mg-repletion on the absorption of Mn are reported as well. To allow discrimination between the effect of Ca or Mg and a possible effect of the inherent addition of counterions (Cl^-) to water and food, the experiments with $CaCl_2$ and $MgCl_2$ were also performed with NaCl in a concentration twice as high as that of $CaCl_2$ and $MgCl_2$.

Materials and methods

Female Swiss Random mice, 3-4 weeks old, were obtained from the Central Institute for the Breeding of Laboratory Animals-TNO, Austerlitz, The Netherlands. IRI-CB diet, a purified diet containing low but adequate levels of minerals and of trace elements [8] (Table 1), and its Ca- and Mg-deplete variant IRI-CB/P were obtained from Hope Farms, Woerden, The Netherlands. The Ca,Mg-deplete diet IRI-CB/P was composed by omitting the relevant minerals (100 µmole Ca/g as $CaCO_3$; 20 µmole Mg/g as $MgSO4.7H_2O$) from the IRI-CB diet during preparation. Carrier-free ^{54}Mn was obtained from New England Nuclear, Boston, Mass., USA.

The following experiments were carried out (see also Table 2 and 3):

Experiment 1. Investigation of the influence of Ca and Mg on the apparent absorption and apparent retention of Mn in mice.

Thirteen groups of 10 mice were fed the Ca,Mg-adequate IRI-CB diet in combination with demineralized drinking water ad libitum during a period of two weeks. After this adaptation period the mice were housed in metabolic cages and fastened for 20 hours. Then ^{54}Mn (as $MnCl_2$) was administered orally, in water (by intragastric intubation) or in food (a freely available portion), or parenterally (by intraperitoneal injection) as follows :

a. Four groups were intubated with 0.3 ml of a ^{54}Mn solution (1 µCi/mouse; 1 µg Mn/ml) in demineralized water as such or supplemented with NaCl, $CaCl_2$, or $MgCl_2$ (50, 25, 25 µmole/ml, resp.). Six hours after ^{54}Mn administration the Ca,Mg-adequate IRI-CB diet was fed again, in combination with demineralized water ad libitum until the end of the experiment.

b. Five other groups were given a portion of food (10 gram per group), thoroughly mixed with 20 µCi ^{54}Mn (carrier-free). Five types of food were used; (1): the Ca,Mg-deplete IRI-CB/P diet; (2), (3) and (4): this IRI-CB/P diet, supplemented with NaCl, $CaCl_2$, or $MgCl_2$ (50, 25 and 25 µmole/g, resp.);

Table 1. Composition of IRI-CB purified diet.

Ingredient	%
Glucose	50.45
Corn starch	15.0
Casein	20.0
Sun flower seed oil	4.0
Fiber (α-cellulose)	5.0
dl Methionine	0.2
Choline chloride	0.3
Minerals [1]	3.95
Trace element mix (NETEM-80) [2]	0.1
Vitamin mix [3]	1.0

[1] Minerals DO : $NaH_2PO_4.2H_2O$, 1.5; KCl, 0.7; $CaCO_3$, 1.0; $MgSO_4.7H_2O$, 0.5; $Na_2SiO_3.9H_2O$, 0.25. [2] Composition in mg/kg diet : Fe_2O_3, 85.9; $MnSO_4.H_2O$, 153.8; ZnO, 25.6; $CuCO_3.Cu(OH)_2$, 17.4; $NiCl_2.6H_2O$, 10.12; NaF, 6.52; $CrCl_3.6H_2O$, 10.25; $SrCl_2.2H_2O$, 3.80; KIO_3, 0.506; $Na_2MoO_4.2H_2O$, 0.504; Na_2SeO_3, 0.329; NH_4VO_3, 0.230; $Na_2HAsO_4.7H_2O$, 0.477; $CoSO_4.7H_2O$, 0.416; $Na_2B_4O_7.10H_2O$, 0.882, glucose to make 1000 mg/kg diet. [3] Composition in IU or mg/kg diet : Retinyl acetate, 16.000 IU; cholecalciferol, 1.400 IU; Menadione, 12; phylloquinone, 2; dl α-tocopheryl acetate, 85; thiamine HCl, 20; riboflavine, 12; pyridoxine HCl, 15; niacin, 40; dl calcium pantothenate, 35; vitamin B 12, 0.05; d biotin, 0.4; folic acid, 8; myo-inositol, 500 mg.

Table 2 Schematic presentation of experiment 1.

Period	Duration	Purpose	Food	Water (exp. 1a,b)	Water (exp. 1c)
1.	14 d	Adaptation	IRI-CB	Demi [1]	Demi
2.	20 h	Fasting	-	Demi	Demi
^{54}Mn dosage [2]					
3.	6 h	Fasting	-	Demi	Demi; +Na; +Ca; +Mg
4.	9 d	Experiment	IRI-CB	Demi	Demi; +Na; +Ca; +Mg

[1] Demineralized water.

[2] Exp. 1a: Orally (by intubation) in water; +Na; +Ca; +Mg . Exp. 1b: Orally (by consumption) in Ca,Mg-deplete food; +Na; +Ca; +Mg; in Ca,Mg-adequate food. Exp. 1c: Parenterally.

Table 3 Schematic presentation of experiment 2.

Period	Duration	Purpose	Food (exp. 2a)	Food (exp. 2b)	Water
1.	14 d	Adaptation	A,B,C,D	D',E,F,G [1]	Demi [2]
2.	20 h	Fasting	-	-	Demi
^{54}Mn dosage [3]					
3.	6 h	Fasting	-	-	Demi
4.	9 d	Experiment	A	D'	Demi

[1] For the composition of diets A-G, see Table 4. [2] Demineralized water.

[3] Orally (by intubation) in water.

(5): the Ca,Mg-adequate IRI-CB diet All diets contained 50 µg Mn/g. The food portions containing ^{54}Mn were made available to the mice for 1.5 hours. Immediately afterwards the intake of each mouse was measured by whole-body counting. Six hours after the start of the 1.5 h feeding period the Ca,Mg-adequate IRI-CB diet was fed again in combination with demineralized water ad libitum until the end of the experiment.

c. The remaining four groups were injected with 0.3 ml of a ^{54}Mn solution (1 µCi/mouse; 1 µg Mn/ml) in acetate buffer (0.05 M, pH 5.6, 0.7% NaCl). As drinking water demineralized water was given as such or supplemented with NaCl, $CaCl_2$, or $MgCl_2$ (50, 25, 25 µmole/ml, resp.), ad libitum until the end of the experiment. Six hours after ^{54}Mn administration the Ca,Mg-adequate IRI-CB diet was fed again ad libitum until the end of the experiment.

Table 4 Composition of mineral-deplete and mineral-replete diets.

Diet [1]		Ca [2] µmole/g	(%)	Mg [3] µmole/g	(%)
A	Ca,Mg-deplete(IRI-CB/P)	—	(—)	—	(—)
B	Mg-deplete	+100	(0.4)	—	(—)
C	Ca-deplete	—	(—)	+20	(0.05)
D [4]	Ca,Mg-adequate	+100	(0.4)	+20	(0.05)
D' [4]	Ca,Mg-adequate (IRI-CB)	100	(0.4)	20	(0.05)
E	Ca-replete	100+250	(1.4)	20	(0.05)
F	Mg-replete	100	(0.4)	20+50	(0.175)
G	Ca,Mg-replete	100+250	(1.4)	20+50	(0.175)

[1] Diet contained 44 µmole Ca/g and 0.7 µmole Mg/g as part of its basic components (analysis by ICPS). [2] As $CaCO_3$. [3] As $MgSO_4.7H_2O$. [4] D equals D'

Experiment 2. Investigation of the influence of the Ca- or Mg-status of mice on the apparent absorption of Mn.

a. Four groups of 10 mice were fed a Ca,Mg-deplete diet (A), or a Mg-deplete diet (B), or a Ca-deplete diet (C), or a Ca,Mg-adequate diet (D) (Table 4), in combination with demineralized drinking water ad libitum during two weeks. After this period the mice were fastened for 20 hours Then each dietary group was intubated with 0.3 ml of a ^{54}Mn solution (1 µCi/mouse; 1 µg Mn/ml) in demineralized water. Six hours after ^{54}Mn administration the Ca,Mg-deplete diet A and demineralized drinking water were given ad libitum until the end of the experiment.

b Four other groups of 10 mice were fed a Ca,Mg-adequate diet (D'), or a Ca-replete diet (E), or a Mg-replete diet (F), or a Ca,Mg-replete diet (G)

(Table 4), in combination with demineralized drinking water ad libitum during two weeks. After this period the mice were fastened for 20 hours. Then each dietary group was intubated with 0.3 ml of a ^{54}Mn solution (1 μCi/mouse, 1 μg Mn/ml) in demineralized water. Six hours after ^{54}Mn administration the Ca,Mg-adequate diet (D') and demineralized water were given ad libitum until the end of the experiment.

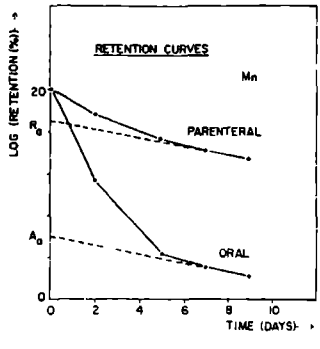


Fig. 1 Determination of apparent absorption A_a and apparent retention R_a, as in the method of Heth and Hoekstra [9].

The retention of ^{54}Mn in the body was measured by whole-body counting during 9 days. The counting was always carried out in the afternoon to eliminate a possible influence of circadian defaecation patterns. The whole-body counter used consisted of two NaI crystals (3"x3"), with their wells (1.5"x2") positioned oppositely, thus forming a cavity in which a mouse could be placed in a reproducible position. Signals from both crystals were summed.

The apparent absorption (A_a) of orally administered ^{54}Mn and the apparent retention (R_a) of parenterally administered ^{54}Mn were calculated by extrapolation of the linear part of the retention curves to the ordinate, as in the method of Heth and Hoekstra [9] (Fig. 1). In some cases the period of measurement (9 days) appeared to be too short for a reliable extrapolation of the line leading to A_a. In such cases, it was assumed that this line was parallel to corresponding extrapolated lines of curves, in which the linear part was reached earlier and therefore an accurate slope could be calculated. Mice which, on basis of their whole-body retention on day 9, were recognized as "outlier" according to the criterion of Chauvenet [10] were not included in these calculations.

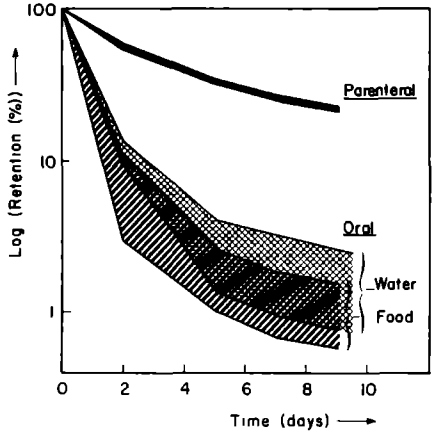


Fig. 2 Areas of comparable mean retention curves of ^{54}Mn, administered in water with mineral additions (exp. 1a, lightly shaded area) and in Ca,Mg-deplete food with mineral additions (exp 1b heavily shaded area), and of ^{54}Mn administered by intraperitoneal injection in combination with drinking water containing minerals (exp 1c black area)

Faeces and urine were collected and measured separately as a check on and supplement to the retention measurements. On the last day of counting all mice were sacrificed. Several organs (in exp 1 liver and kidneys, in exp 2: liver, kidneys, pancreas and heart) were collected and their ^{54}Mn content measured using an auto-gamma scintillation spectrometer (Packard 5120)

Results

Fig 2 shows the areas that included the mean retention curves of ^{54}Mn, administered to mice orally (in water or in food) and parenterally (by intraperitoneal injection) (exp 1) Fig. 3 shows the apparent absorption A_a of ^{54}Mn, calculated from these curves according to the method shown in Fig 1 In general A_a was somewhat lower for food than for water, as can also be seen from the position of the areas in Fig. 2 Ca and Mg strongly reduced A_a both in water and in food NaCl, added to water and food to account for a possible effect of the addition of chloride anions, showed no statistically significant influence on A_a Fig. 4 shows the apparent retention R_a, calculated from the retention curves of intraperitoneally injected ^{54}Mn (Fig 2) again according to the method shown in Fig 1 Addition of Na, Ca or Mg to the drinking water did not have a significant effect on R_a

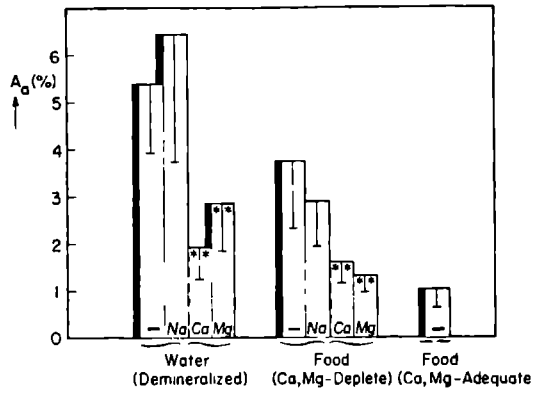


Fig. 3 Apparent absorption A_a of ^{54}Mn from an intubated solution (exp. 1a: left) and from Ca,Mg-deplete food (exp. 1b: right) as influenced by addition of minerals (Na, Ca and Mg : 50, 25 and 25 µmole/ml solution and 50, 25 and 25 µmole/g food, respectively) and from Ca,Mg-adequate food (exp. 1b: most right). Data are presented as mean with one standard deviation at the lower side. Statistical comparison: ** = significantly different from control (-) and from Na addition (Na) by Student's t-test ($P<0.05$).

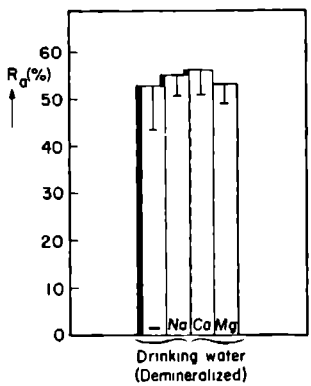


Fig. 4 Apparent retention R_a of intraperitoneally injected ^{54}Mn as influenced by addition of minerals (Na, Ca and Mg : 50, 25 and 25 µmole/ml, respectively) to the drinking water (exp. 1c). Data are presented as mean with one standard deviation at the lower side. No significant differences were found.

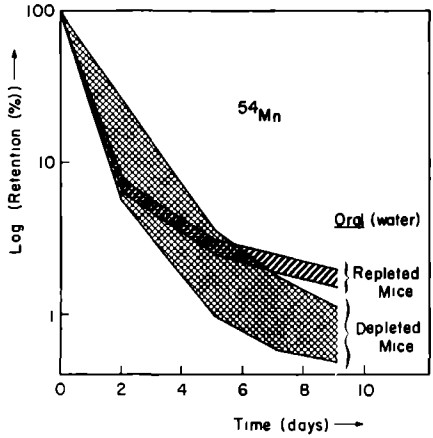


Fig. 5 Areas of comparable mean retention curves of ^{54}Mn, administered to mice fed Ca,Mg-adequate or Ca- or Mg-deplete diets (exp 2a light shaded area) and to mice fed Ca,Mg-adequate or Ca- or Mg-replete diets (exp. 2b dark shaded area) during 2 weeks prior to dosage. The former groups of mice were fed a Ca,Mg-deplete diet, the latter a Ca,Mg-adequate diet during the period of measurement after dosage.

As shown in Fig. 5, the retention curves of ^{54}Mn administered to mice with varied mineral status (exp 2) had the usual shape for the mice fed mineral-replete diets A-C and their corresponding control group fed diet D (exp. 2b), but were strongly different for mice fed mineral-deplete diets E-G and their corresponding control group fed diet D' (exp 2a). Because a deviating curve shape was not only found for the experimental groups, but also for the control group, it was concluded that this effect was caused by the feeding of the Ca,Mg-deplete diet A to the mice after ^{54}Mn administration (see methods). As a result of this deviating curve shape the method used for determination of the apparent absorption A_a (Fig 1) could not be applied. Particularly the part of the retention curve measured after day 2, i.e., the 2nd day after ^{54}Mn administration, seemed to be affected. Therefore, it was decided to use the retention on day 2 (R_2) as a measure for absorption, assuming that at that time intestinal passage of unabsorbed ^{54}Mn was completed. This use had the sole purpose of detecting possible effects of the mineral status on ^{54}Mn absorption. Fig. 6 shows this R_2 for the mice fed mineral deplete and -replete diets with their corresponding control groups. The control groups have the same value for R_2, which indicates that R_2 was not significantly affected by the difference in diets fed after dosage. R_2 was strongly

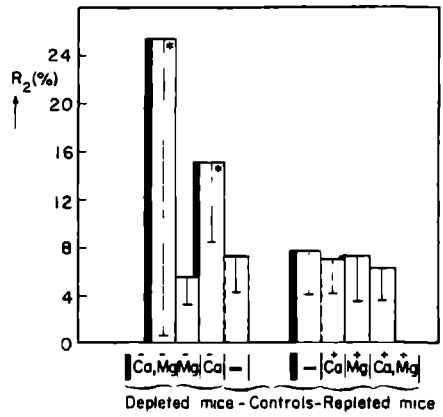


Fig. 6 Retention on day 2 (R_2) of ^{54}Mn from an intubated solution as influenced by feeding of Ca- or Mg-deplete diets (exp. 2a: left) or Ca- or Mg-replete diets (exp. 2a: right) during 2 weeks prior to ^{54}Mn administration. The mice were fastened from 20 hours before dosage until 6 hours after dosage. Data are presented as mean with one standard deviation at the lower side. Statistical comparison: * = significantly different from control (-) by Student's t-test ($P<0.05$).

increased, however, in the mice fed the Ca-deplete diet C or the Ca,Mg-deplete diet A, but not in mice fed the Mg-deplete diet B.

The ^{54}Mn content in liver and kidneys (expressed as % of the whole-body content on the day of sacrifice) of the mice used in experiment 1 (Table 5) did not show a relation to the apparent absorption A_a or apparent retention R_a. However, as shown in Fig. 7, in experiment 2 significant differences were observed for the ^{54}Mn contents of the organs of the Ca-deprived mice compared to the control mice. Particularly the ^{54}Mn content of the pancreas was strongly reduced in the mice fed the Ca,Mg-deplete diet after ^{54}Mn administration.

Discussion

When plotted semi-logarithmically, the retention curve of parenterally administered ^{54}Mn in mice (Fig. 1) shows, within the period of measurement, a biphasic elimination of ^{54}Mn. The rapid phase, represented by the non-linear part of the curve, reflects endogenous excretion of ^{54}Mn that is rapidly removed from the circulation, mainly by the liver that eliminates Mn with the bile [11], but also by various parts of the intestine that excrete Mn

Table 5 ^{54}Mn content of liver and kidneys of mice, expressed as % of the whole-body content on the day of sacrifice, as related to the apparent absorption (A_a) or retention (R_a).

^{54}Mn absorption from water (exp. 1a) :

Mineral added [1]	A_a (%)	Liver (%)	Kidneys (%)
-	5.4±1.4	19.5±2.0	6.2±0.8
Na	6.4±2.7	19.8±2.9	6.7±0.7
Ca	1.9±0.7 *	19.6±3.5	6.2±0.5
Mg	2.8±1.0 *	22.7±3.1*	6.3±0.8

^{54}Mn absorption from Ca,Mg-deplete and Ca,Mg-adequate food (exp. 1b) :

Mineral added [1]	A_a (%)	Liver (%)	Kidneys (%)
-	3.7±1.4	20.1±2.7	6.6±0.9
Na	2.9±0.9	20.1±3.4	6.0±0.7
Ca	1.6±0.4 *	20.7±3.4	5.8±1.1
Mg	1.3±0.4 *	16.5±1.7*	5.8±0.8
Adeq.	1.0±0.4 *	20.1±4.1	6.8±1.1

^{54}Mn retention after intraperitoneal injection (exp. 1c) :

Mineral added [2]	Ra (%)	Liver (%)	Kidneys (%)
-	52.8±9.4	14.4±3.5	5.7±0.5
Na	55.2±4.5	16.1±1.7	6.2±0.7
Ca	56.3±5.4	15.7±3.3	6.0±0.6
Mg	53.3±4 2	17.8±1.2*	6.0±0.9

*) Signuficantly different from control (-) by Student's t-test ($P<0.05$)
[1] Mineral added to the ^{54}Mn dose. [2] Mineral added to the drinking water.

with the digestive juice [12] and at higher doses by the pancreas that excretes Mn with the pancreatic juice [13]. The slow phase, represented by the linear part of the curve, reflects the steady turnover (with a constant biological half-life, $t_{1/2}$= 8 4 days) and endogenous excretion of ^{54}Mn that has been equilibrated with the exchangeable Mn body pools [14,15]

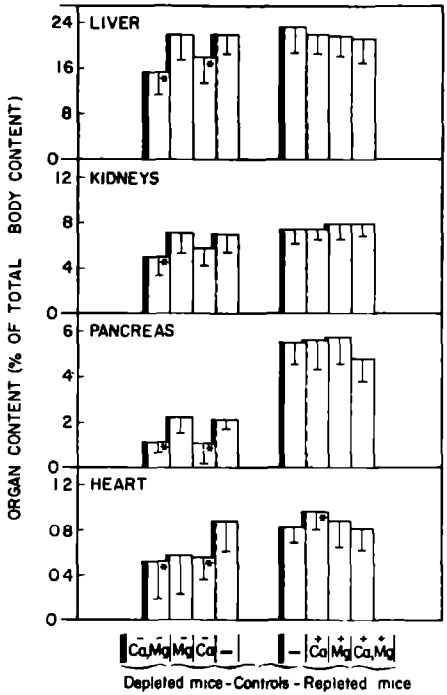


Fig. 7 ^{54}Mn content of liver, kidneys, pancreas and heart of mice, expressed as % of the whole-body content on the day of sacrifice; influence of mineral depletion and repletion (exp. 2). Data are presented as mean with one standard deviation at the lower side. Statistical comparison: as in Fig. 6.

In addition to this biphasic elimination the retention curve of orally administered ^{54}Mn (Fig. 1) contains a first rapid phase reflecting the elimination of unabsorbed ^{54}Mn from the gut with the faeces. This phase, which overlaps the first rapid phase of elimination of absorbed ^{54}Mn, may represent two fractions, viz., ^{54}Mn that has never been bound to mucosal cells and is excreted with undigested food components, and ^{54}Mn that has been bound to mucosal cells, but has not been taken up into the circulation. The latter fraction of ^{54}Mn is lost by desquamation of the mucosal cells and its excretion will be retarded with respect to the excretion of the former fraction. As both forms of excretion probably overlap, it is difficult to distinguish

between them.

The fraction of the oral or parenteral dose which is eliminated with a constant biological half-life represents a specific part of the dose, i.e., the part equilibrated with body stores. This fraction is found by extrapolation of the linear part of the retention curve to time 0 (Fig. 1) and is called apparent absorption A_a in case of oral administration and apparent retention R_a in case of parenteral administration [16]. Though A_a and R_a are not equal to the true absorption and retention, they can be used to describe changes in these processes A_a and R_a are used here to describe the effects of Ca and Mg on the absorption of Mn from water and from food and, when added to the drinking water, on the retention of Mn, injected intraperitoneally.

The strong decrease of A_a observed when Ca or Mg were added to the ^{54}Mn dose (Fig. 3), both when this dose was given as an aqueous solution and when it was mixed with purified food (without free Ca- and Mg-salts), indicates that both minerals reduce the absorption of Mn from the gastrointestinal tract. The interaction between Ca, Mg and Mn could occur in the lumen, on the mucosal cell membrane, or inside the mucosal cell. In the lumen coprecipitation of Mn could occur when insoluble complexes of Ca or Mg, e.g., with components from the food (phosphate, phytate, fiber) are formed. Such coprecipitation would reduce the availability of Mn for absorption. In swine [17], poultry [18] and calves [19] excessive intakes of Ca and phosphate have been known to accentuate the dietary requirements for Mn. Phytate has been shown to reduce the availability of Mn in rats [20].

In the present study Ca and Mg reduced the absorption of Mn not only in the presence of food, but also when Mn was administered in water. Therefore, another effect could occur in addition, e.g., a competition for receptor and carrier proteins of the mucosal cell. The uptake of Mn into the mucosal cell and the transport of Mn to the circulation are reported to be two kinetically different steps in the absorption pathway [21] A competition could exist for one of these steps and possibly for both. A similar process of mucosal uptake and transport has been found for Fe and Co [21,22] Thomson and Valberg [21] have shown that the absorption pathways of these three metals are strongly interrelated. Competition between Mn, Fe and Co has been observed both for uptake into the mucosal cell and for transport to the circulation [23,24] It is not known, however, which proteins are involved in these absorption processes.

The concept of a competition between Ca and Mg on the one hand and Mn on the other hand for a common step in the absorption pathway is supported by the observation that the absorption of Co from water is reduced by Ca and Mg in a similar way [16] This indicates that the common step concerned might involve Co and possibly also Fe However, a similar effect of Ca and Mg on Fe absorption is not known

The retention curves of orally administered ^{54}Mn, obtained in experiment

2 (Fig. 5), show a different shape for the mice fed the Ca,Mg-deplete diet A after dosage. As shown in Fig. 3, the apparent absorption of Mn from the Ca,Mg-deplete diet A (3.7%) is about 4 times as high as from the Ca,Mg-adequate diet D' (1.0%). Therefore, the mice fed diet A during 9 days following ^{54}Mn administration, probably absorbed 4 times as much stable Mn from the food during this period as the mice fed diet D' during the same period. According to Mariënfeld and Collins [3] homeostasis of Mn is very efficient and no Mn accumulation seems to occur. The turnover of ^{54}Mn in the mice fed diet A after dosage could therefore have been increased by the flow of stable Mn absorbed from this diet. Several authors [14,15,25] have shown that the turnover of ^{54}Mn is likewise increased by a high dietary Mn intake.

Lassiter et al. have found an increased turnover of ^{54}Mn in rats fed a low Ca diet; they concluded that "the general assumption that Mn metabolism is affected only in the gut should be invalid" [6]. Our results indicate that this general assumption is still valid. It is recognized, however, that ^{54}Mn turnover not only depends on the level of dietary Mn intake, as has been shown long ago by Cotzias and Greenough [14], but also on the fraction of dietary Mn actually absorbed. This fraction appears to be increased in rats and mice fed a low Ca diet.

As may be concluded from the retention on the 2nd day after dosage (R_2) (Fig. 6), ^{54}Mn absorption was strongly increased in mice fed a Ca-deplete diet (A or C) prior to dosage. An explanation for this effect may be that in Ca-deprived mice the endogenous excretion of Ca, which mainly occurs in the faeces [26], could be reduced and that as a consequence the intraluminal concentration of Ca in the gut at the moment of ^{54}Mn administration could be lower in Ca-deprived mice than in Ca-adequate ones. If this endogenous Ca could also compete with Mn for a common step in the absorption pathway, then a low intraluminal Ca content in Ca-deprived mice could favour Mn absorption. In Mg-deprived mice the endogenous excretion of Mg should not play a role, because Mg is primarily excreted in the urine [27]. Indeed, no significant increase of ^{54}Mn absorption was observed in the mice fed the Mg-deplete diet (B) (Fig. 6).

In experiment 1 the relative uptake of ^{54}Mn in liver and kidneys was found to be independent of the extent of ^{54}Mn absorption (Table 4) The effect of Mg on the ^{54}Mn content in the liver was inconsistent. Feeding diet A after dosage (exp. 2) had a remarkable effect on the ^{54}Mn content in the pancreas (Fig. 7); the low ^{54}Mn content indicates an increased pancreatic turnover of Mn, related to the increased Mn turnover in the whole body as described earlier. In the Ca-deprived mice the ^{54}Mn content of all measured organs was decreased compared to the control group. It is not clear whether these effects reflect differences in absorption and turnover of ^{54}Mn or biochemical changes of organ function due to Ca-deprivation, as is suggested by Lassiter et al. [6].

Table 6 The influence of minerals on ^{54}Mn absorption from water (exp 1a) and from food (exp. 1b), and of mineral depletion or repletion during 2 weeks (exp. 2) on ^{54}Mn absorption from water. [1]

	Water	Food	Depletion		Repletion	
Na [2]	-	-	-Ca	↑	+Ca	-
Ca [3]	↓	↓	-Mg	-	+Mg	-
Mg [3]	↓	↓	-Ca,Mg	↑	+Ca,Mg	-

[1] "↓" and "↑" mean a decrease or increase of apparent absorption A_a under the conditions described [2] 50 µmole/ml or g. [3] 25 µmole/ml or g.

As is shown in Table 6, the results can be classified into two major effects. One effect is a reduction of Mn absorption by the presence of Ca or Mg in the drinking water or the food. The other effect is an increase of the Mn absorption by chronic Ca-deprivation. Concerning public health, the data indicate that the uptake of Mn may be higher in soft water regions than in hard water regions due to differences in hardness of the drinking water. An additional increase of the Mn absorption may occur, when the Ca intake from the diet is marginal. Marienfeld and Collins [3] have suggested that increased Mn uptake may have various detrimental effects on health. However, the homeostatic potential of the body towards Mn might be sufficient to manage large differences in Mn uptake. To eliminate doubts, attention should be payed to Mn in epidemiological investigations correlating drinking water quality to health aspects.

Acknowledgements

The authors wish to acknowledge Prof. C.J. Marienfeld from the University of Missouri-Columbia, Columbia, Missouri, USA, for his encouragement to include manganese in the research program, which has ultimately led to this paper. Prof. J.P.W. Houtman and Dr. J.H.M. Van Tongeren are acknowledged for critical review of the manuscript. Mr. C.A. Witteman is thanked for technical assistance.

References

1. National Research Council, (1980) In: Recommended Dietary Allowances, 9th ed., National Academy of Sciences, Washington D.C., 1980, pp. 154-156.
2. Underwood, E. (1977) In: Trace elements in human and animal nutrition, pp. 189-190, 4th ed., Academic Press, N.Y.

3. Marienfeld, C.J. and Collins, M. (1981) The ebb and flow of manganese: A possible pathogenic factor in birth defects, cancer and heart disease. Tr. Subst. Environm. Hlth 15, 3-20.
4. Schroeder, H.A., Nason, A.P. and Balassa, J.J. (1967) Trace metals in rat tissues as influenced by calcium in water. J.Nutr. 93, 331-336.
5. Ingols, R.S. and Craft, T.F. (1976) Analytical note - hard- vs soft-water effects on the transfer of metallic ions from intestine. J. Am. Water Works Ass. 68, 209-210.
6. Lassiter, J.W., Miller, W.J., Pate, F.M. and Gentry, R.P. (1972) Effect of dietary calcium and phosphorus on ^{54}Mn metabolism following single tracer intraperitoneal and oral doses in rats. Proc.Soc.exp.Biol.Med. 139, 345-348.
7. Pond, W.G., Walker, Jr., E.F., Kirtland, D. and Rounsaville, T. (1978) Effect of dietary Ca, Cu and Zn level on body weight gain and tissue mineral concentrations of growing pigs and rats. J.Anim.Sci. 47, 1128-1134.
8. Van Barneveld, A.A. and Morse, H. (1982) A new diet for trace element research with mice and rats. Z. Versuchstierk. 24, 24. IRI-Rapport 133-81-02, Delft, The Netherlands.
9. Heth D.A. and Hoekstra, W.G. (1965) Zinc-65 absorption and turnover in rats. I. A procedure to determine zinc-65 absorption and the antagonistic effect of calcium in a practical diet. J. Nutr. 85, 367-374.
10. De Soete, D., Gijbels, R. and Hoste, J. (1972) In: Neutron Activation Analysis, Chemical Analysis vol. 34, ed. P.J. Elving and J.M. Kolthof, Wiley-Interscience, London, 1972, pp. 505-506.
11. Papavasiliou, P.S., Miller, S.T. and Cotzias, G.C. (1966) Role of liver in regulating distribution and excretion of manganese. Am.J.Physiol. 211, 211-216.
12. Bertinchamps, A.J., Miller, S.T. and Cotzias, G.C. (1966) Interdependence of routes excreting manganese. Am.J.Physiol. 211, 217-224.
13. Burnett, W.T., Bigelow, R.R., Kimble, A.W. and Sheppard, C.W. (1952) Am.J.Physiol. 168, 620-625.
14. Cotzias, G.C. and Greenough, J.J. (1958) The high specificity of the manganese pathway through the body. J.Clin.Invest. 37, 1298-1305.
15. Britton, A.A. and Cotzias, G.C. (1966) Dependence of manganese turnover on intake. Am. J. Physiol. 211, 203-206.
16. Van Barneveld, A.A., Van den Hamer, C.J.A. and Houtman, J.P.W. (1982) Drinking water hardness, trace elements and cardiovascular diseases: Influence of Ca and Mg on metabolism of Cd, Co, Cu, Zn and Se in mice. Tr.Subst.Environ.Hlth 16, 196-204.
17. Miller, R.C., Keith, T.B., McCarty, M.A. and Thorp, W.T.S. (1940) Manganese as a possible factor influencing the occurrence of lameness in pigs. Proc.Soc.exp.Biol.Med. 45, 50-51.
18. Schaible, P.J. and Bandemer, S.A. (1942) The effect of mineral supple-

ments on the availability of manganese. Poultry Sci. 21, 8-14.

19. Hawkins, G.E., Wise, G.H., Matrone, G. and Waugh, R.H. (1955) Manganese in the nutrition of young dietary cattle fed different levels of calcium abd phosphorus. J.Dairy Sci. 38, 536-547.
20. Davies, N.T. and Nightingale, R. (1975) The effects of phytate on intestinal absorption and secretion of zinc, and whole-body retention of Zn, copper, iron and manganese in rats. Br.J.Nutr. 34, 243-258.
21. Thomson, A.B.R. and Valberg, L.S. (1972) Intestinal uptake of iron, cobalt, and manganese in the iron-deficient rat. Am.J.Physiol. 223, 1327-1329.
22. Becker, G., Huebers, H. and Rummel, W. (1979) Intestinal absorption of cobalt and iron: Mode of interaction and subcellular distribution. Blut 38, 397-406.
23. Thomson, A.B.R., Olatunbosun, D. and Valberg, L.S. (1971) Interrelation of intestinal transport system for manganese and iron. J.Lab.Clin.Med. 78, 642-655.
24. Flanagan, P.R., Haist, J. and Valberg, L.S. (1980) Comparative effects of iron deficiency induced by bleeding and a low-iron diet on the intestinal absorptive interactions of iron, cobalt, manganese, zinc, lead and cadmium. J.Nutr. 110, 1754-1763.
25. Abrams, E., Lassiter, J.W., Miller, W.J., Neathery, M.W., Gentry, R.P. and Scarth, R.D. (1976) Absorption as a factor in manganese homeostasis. J.Anim.Sci. 42, 630-636.
26. Roith, D. le, and Pimstone, B. (1973) The use of simultaneously administered ^{47}Ca and ^{45}Ca in the study of calcium kinetics in the rat. Horm.Metab.Res. 5, 118-123.
27. Ebel, H. and Günther, T. (1980) Magnesium metabolism: a review. J.Clin.Chem.Clin.Biochem. 18, 257-270.

5.4. PAPER IX

INFLUENCE OF CA AND MG ON METABOLISM OF PB AND CD IN MICE

A.A. Van Barneveld and C.J.A. Van den Hamer

ABSTRACT The influence of high but not extreme concentrations of Ca and Mg (25 µmole/ml) on the apparent absorption and retention of ^{203}Pb and ^{115m}Cd from water was investigated in mice. Ca reduced the absorption of Pb from an intubated solution by 62% ($P<0.10$). The absorption of Cd was only slightly reduced by Ca (22%) and the effect was not significant. Mg showed no effect on Pb and Cd absorption. The retention of intraperitoneally injected Pb and Cd was not influenced by addition of Ca or Mg to the drinking water. Feeding of a Ca-deplete diet during two weeks prior to dosage stimulated the absorption of Pb from water by more than 100% ($P<0.01$) and the absorption of Cd from water by 17% ($P<0.05$). Feeding of a Ca-replete diet did not influence the absorption of Pb or Cd, but faecal Cd excretion seemed to be reduced. Feeding of Mg-deplete or Mg-replete diets did not show any effect on Pb and Cd metabolism. It is concluded that in soft water regions the uptake of Pb from the drinking water could be increased because of the absence of Ca in the drinking water, particularly when dietary Ca intake is low. This relationship between drinking water hardness and absorption of Pb could be important in view of general public health.

Introduction

The presence of the toxic elements Pb and Cd in drinking water and their effects on human health are subject of much concern to drinking water producers and public health officers. General agreement on maximum permissible concentrations in drinking water has not been achieved. Allowed concentrations in drinking water vary in different countries from 1 to 50 µg/l for Cd and from 30 to 100 µg/l for Pb [1].

The concentration of these metals in the drinking water may partly depend on the hardness of water. Soft water seems to promote solubilization of Pb and Cd from water pipes and solder joints between water pipes [2-4], thus increasing the concentration of these metals in drinking water. As concerned Pb, this point is controversial because a recent report showed a positive correlation between water hardness and Pb concentration in drinking water in The Netherlands [5].

From several studies of Pb and Cd metabolism in laboratory animals it might be concluded that water hardness could also have a physiological effect on the absorption of Pb and Cd from drinking water in the gut. In the rat Ca has been shown to decrease the absorption of Pb from water [6-8]. In the

chick Ca decreased the absorption of Cd from water [9]. Only one of these reports [6] suggested a relation between drinking water hardness and heavy metal absorption in man.

The present study reports effects of both Ca and Mg (as $CaCl_2$ and $MgCl_2$) on the absorption in mice of Pb and Cd from water. Effects of Ca- or Mg-depletion and of Ca- or Mg-repletion on the absorption of Pb and Cd are also reported. To allow discrimination between the effect of Ca or Mg and a possible effect of the inherent addition of counterions (Cl^-) to water, the experiments with $CaCl_2$ and $MgCl_2$ were also performed with NaCl in a concentration twice as high as that of $CaCl_2$ and $MgCl_2$.

Materials and methods

Female Swiss Random mice, 3-4 weeks old, were obtained from the Central Institute for the Breeding of Laboratory Animals-TNO, Austerlitz, The Netherlands. IRI-CB diet, a purified diet containing low but adequate levels of minerals and of trace elements [10] (Table 1), and its Ca- and Mg-deplete variant IRI-CB/P were obtained from Hope Farms, Woerden, The Netherlands. The Ca,Mg-deplete diet IRI-CB/P was composed by omitting the relevant minerals (100 µmole Ca/g as $CaCO_3$; 20 µmole Mg/g as $MgSO_4.7H_2O$) from the IRI-CB diet during preparation. ^{115m}Cd (0.2 µCi/g Cd) and carrier-free ^{203}Pb were obtained from New England Nuclear, Boston, Mass., USA.

The following experiments were carried out (see also Table 2 and 3).

Experiment 1. Investigation of the influence of Ca and Mg on the apparent absorption and apparent retention of Pb and Cd in mice.

Eight groups of 20 mice (10 for the ^{203}Pb experiment and 10 for the ^{115m}Cd experiment) were fed the Ca,Mg-adequate IRI-CB diet in combination with demineralized drinking water ad libitum during two weeks. After this adaptation period the mice were housed in metabolic cages and fasted for 20 hours. Then ^{203}Pb or ^{115m}Cd was administered by intragastric intubation or by intraperitoneal injection (as $PbCl_2$ and $CdCl_2$, resp.) as follows:

a. Four groups were intubated with 0.3 ml of a ^{203}Pb or ^{115m}Cd solution (1 µCi/mouse, 1 µg Pb/ml or 15 µg Cd/ml) in demineralized water as such or supplemented with NaCl, $CaCl_2$, or $MgCl_2$ (50, 25, 25 µmole/ml, resp.). As drinking water demineralized water was given ad libitum until the end of the experiment.

b. The other four groups were injected with 0.3 ml of a ^{203}Pb or ^{115m}Cd solution (1 µCi/mouse, 1 µg Pb/ml or 15 µg Cd/ml) in acetate buffer (0.05 M, pH 5.6, 0.7% NaCl). As drinking water demineralized water was given as such or supplemented with NaCl, $CaCl_2$, or $MgCl_2$ (50, 25, 25 µmole/ml, resp.), ad libitum until the end of the experiment.

Six hours after isotope administration the Ca,Mg-adequate IPI-CB diet was fed again to all groups ad libitum until the end of the experiment.

Table 1. Composition of IRI-CB purified diet.

Ingredient	%
Glucose	50.45
Corn starch	15.0
Casein	20.0
Sun flower seed oil	4.0
Fiber (α-cellulose)	5.0
dl Methionine	0.2
Choline chloride	0.3
Minerals [1]	3.95
Trace element mix (NETEM-80) [2]	0.1
Vitamin mix [3]	1.0

[1] Minerals (%) : $NaH_2PO_4.2H_2O$, 1.5; KCl, 0.7; $CaCO_3$, 1.0; $MgSO_4.7H_2O$, 0.5; $Na_2S_2O_3.9H_2O$, 0.25. [2] Composition in mg/kg diet : Fe_2O_3, 85.9; $MnSO_4.H_2O$, 153.8, ZnO, 25.6; $CuCO_3.Cu(OH)_2$, 17.4; $NiCl_2.6H_2O$, 10.12; NaF, 5.52; $CrCl_3.6H_2O$, 10.25; $SnCl_2.2H_2O$, 3.80; KIO_3, 0.506; $Na_2MoO_4.2H_2O$, 0.504; Na_2SeO_3, 0.329; NH_4VO_3, 0.230; $Na_2HAsO_4.7H_2O$, 0.477; $CoSO_4.7H_2O$, 0.416; $Na_2B_4O_7.10H_2O$, 0.882; glucose to make 1000 mg/kg diet. [3] Composition in IU or mg/kg diet : Retinyl acetate, 16,000 IU; cholecalciferol, 1,400 IU. Menadione, 12; phylloquinone, 2, dl α-tocopheryl acetate, 85; thiamine HCl, 20; riboflavine, 12; pyridoxine HCl, 15; niacin, 40; dl calcium pantothenate, 35; vitamin B 12, 0.05; d biotin, 0.4; folic acid, 8; myo-inositol, 500 mg.

Table 2 Schematic presentation of experiment 1.

Period	Duration	Purpose	Food	Water (exp. 1a)	Water (exp. 1b)
1.	14 d	Adaptation	IRI-CB	Demi [1]	Demi
2.	20 h	Fasting	-	Demi	Demi
^{203}Pb, ^{115m}Cd dosage [2]					
3.	6 h	Fasting	-	Demi	Demi; +Na; +Ca; +Mg
4.	9 d	Experiment	IRI-CB	Demi	Demi; +Na; +Ca; +Mg

[1] Demineralized water.

[2] Exp. 1a: Orally (by intubation) in water; +Na; +Ca; +Mg . Exp. 1b: Parenterally.

Table 3 Schematic presentation of experiment 2.

Period	Duration	Purpose	Food (exp. 2a)	Food (exp. 2b)	Water
1.	14 d	Adaptation	A,B,C,D	D',E,F,G [1]	Demi [2]
2.	20 h	Fasting	-	-	Demi
^{203}Pb, ^{115m}Cd dosage [3]					
3.	6 h	Fasting	-		Demi
4.	9 d	Experiment	A	D'	Demi

[1] For the composition of diets A-G, see Table 4. [2] Demineralized water.

[3] Orally (by intubation) in water.

Table 4. Composition of mineral-deplete and mineral-replete diets.

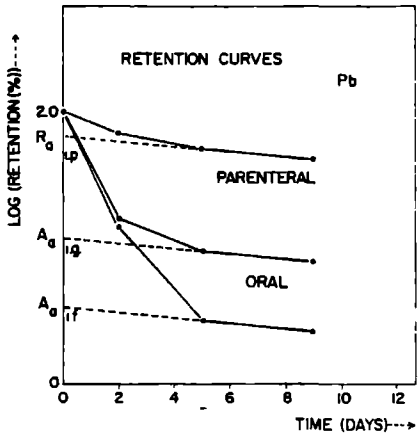
Diet [1]		Ca [2] µmole/g	(%)	Mg [3] µmole/g	(%)
A	Ca,Mg-deplete(IRI-CB/P)	—	(—)	—	(—)
B	Mg-deplete	+100	(0.4)	—	(—)
C	Ca-deplete	—	(—)	+20	(0.05)
D [4]	Ca,Mg-adequate	+100	(0.4)	+20	(0 05)
D' [4]	Ca,Mg-adequate (IRI-CB)	100	(0.4)	20	(0.05)
E	Ca-replete	100+250	(1.4)	20	(0 05)
F	Mg-replete	100	(0.4)	20+50	(0 175)
G	Ca,Mg-replete	100+250	(1.4)	20+50	(0.175)

[1] Diet contained 44 µmole Ca/g and 0.7 µmole Mg/g as part of its basic components (analysis by ICPS). [2] As $CaCO_3$. [3] As $MgSO_4 7H_2O$ [4] D equals D'

Experiment 2. Investigation of the influence of the Ca- or Mg-status of mice on the apparent absorption of Pb and Cd.

a. Four groups of 20 mice (10 for the ^{203}Pb experiment and 10 for the ^{115m}Cd experiment) were fed a Ca,Mg-deplete diet (A), or a Ca-deplete diet (B), or a Mg-deplete diet (C), or a Ca,Mg-adequate diet (D) (Table 4), in combination with demineralized drinking water ad libitum during two weeks. After this period the mice were fasted for 20 hours Then each dietary group was intubated with 0.3 ml of a ^{203}Pb or ^{115m}Cd solution (1 µCi/mouse, 1 µg Pb/ml or 15 µg Cd/ml) in demineralized water Six hours after isotope administration the Ca,Mg-deplete diet (A) and demineralized drinking water were given ad libitum until the end of the experiment.

b Four other groups of 20 mice (10 for the ^{203}Pb experiment and 10 for the ^{115m}Cd experiment) were fed a Ca,Mg-adequate diet (D'), or a Ca-replete diet (E), or a Mg-replete diet (F), or a Ca,Mg-replete diet (G) (Table 4), in combination with demineralized drinking water ad libitum during two weeks After this period the mice were fasted for 20 hours Then each dietary group was intubated with 0.3 ml of a ^{203}Pb or ^{115m}Cd solution (1µCi/mouse, 1 µg Pb/ml or 15 µg Cd/ml) in demineralized water Six hours after isotope administration the Ca,Mg-adequate diet (D') and demineralized water were given ad libitum until the end of the experiment.



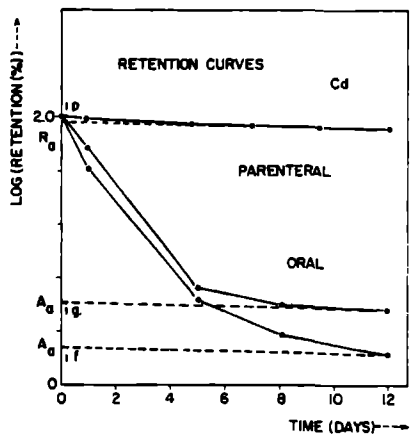


Fig. 1 Determination of apparent absorption A_a and apparent retention R_a according to the method of Heth and Hoekstra [11].

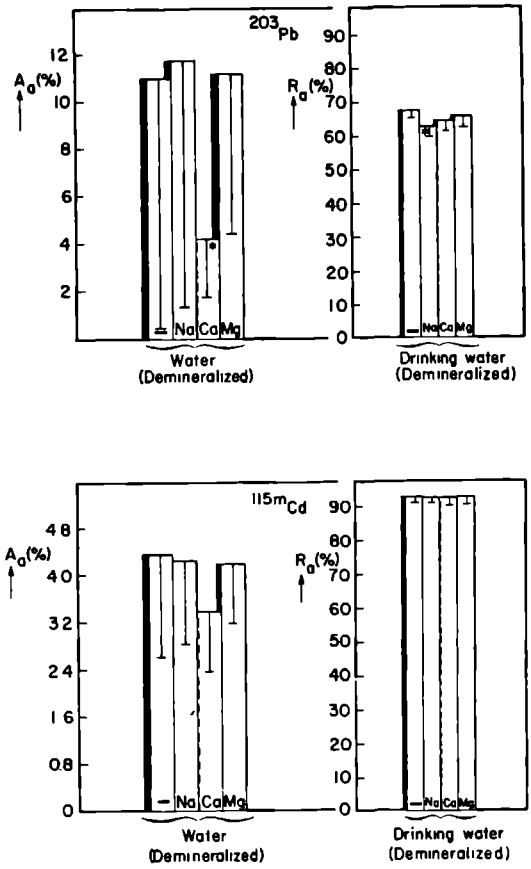


Fig. 2 and Fig. 3 Apparent absorption A_a of ^{203}Pb and ^{115m}Cd from an intubated solution (exp. 1a: left) and apparent retention R_a of intraperitoneally injected ^{203}Pb and ^{115m}Cd (exp. 1b: right) as influenced by addition of minerals (Na, Ca and Mg : 50, 25 and 25 µmole/ml, respectively) to the intubated solution (A_a) and to the drinking water (R_a), respectively. Data are presented as mean with one standard deviation at the lower side. Statistical comparison: * = significantly different from control (-) and from Na addition (Na) by Student's t-test (Fig. 2: $P<0.10$; Fig. 3: $P<0.05$).

The retention of the radioisotopes in the body was measured by whole-body counting during 9-12 days. The counting was always carried out in the afternoon to eliminate a possible influence of circadian defaecation patterns. The whole-body counter used consisted of two NaI crystals (3"x3"), with their wells (1.5"x2") positioned oppositely, thus forming a cavity in which a mouse could be placed in a reproducible position. Signals from both crystals were summed.

The apparent absorption (A_a) of orally administered ^{203}Pb and ^{115m}Cd and the apparent retention (R_a) of parenterally administered ^{203}Pb and ^{115m}Cd were calculated by extrapolation of the linear part of the retention curves to the ordinate, as in the method of Heth and Hoekstra [11] (Fig. 1). In some cases the period of measurement (9-12 days) appeared to be too short for a reliable extrapolation of the line leading to A_a. In such cases, it was assumed that this line was parallel to corresponding extrapolated lines of other curves in which the linear part was reached earlier and therefore an accurate slope could be measured. Mice which, on basis of their whole-body retention on day 9, were recognized as "outlier" according to the criterion of Chauvenet [12] were not included in these calculations. On the last day of counting all mice were sacrificed. In the ^{203}Pb experiments two organs (liver and kidneys) and also the two hind legs were collected and their ^{203}Pb content measured using an auto-gamma scintillation spectrometer (Packard 5120).

Results and discussion

Fig. 2 shows the apparent absorption A_a of ^{203}Pb from an intubated solution and the apparent retention R_a of intraperitoneally injected ^{203}Pb. A_a of Pb was reduced in the presence of Ca, but not of Mg, in the intubated solution (62%; $P<0.10$). A similar effect of Ca has also been found in rats [6-8], but not in chicks [13]. According to Barton et al. [7], Ca and Pb may compete for two proteins in the cytosol of the mucosal cell, viz., calcium binding protein (CaBP) and a non-specified protein with a high molecular weight. This competition may determine the degree of absorption of Pb. Hilburn et al. [8] have reported that in experiments with everted gut sacs of rats a total absence of Ca allows extracellular Pb-transport by opening of the tight junctions and desmosomes between the mucosa cells. In their experiments this effect was abolished by an intraluminal Ca concentration of only 0.6 µmole/mL. The presence of a minimal amount of Ca seems to reduce Pb absorption to a level, which is not further reduced by Ca addition.

Barltrop and Khoo [14] have shown that addition of Ca (0.7%) or Mg (0.065%) to Ca,Mg-poor food reduced the absorption of Pb from the food in rats. Fine et al. [15] have found similar results for addition of Mg (0.012%) to Ca,Mg-poor food of dogs. The formation of complexes of Pb with Ca or Mg and food components like phosphate might play a role. Such complexes are

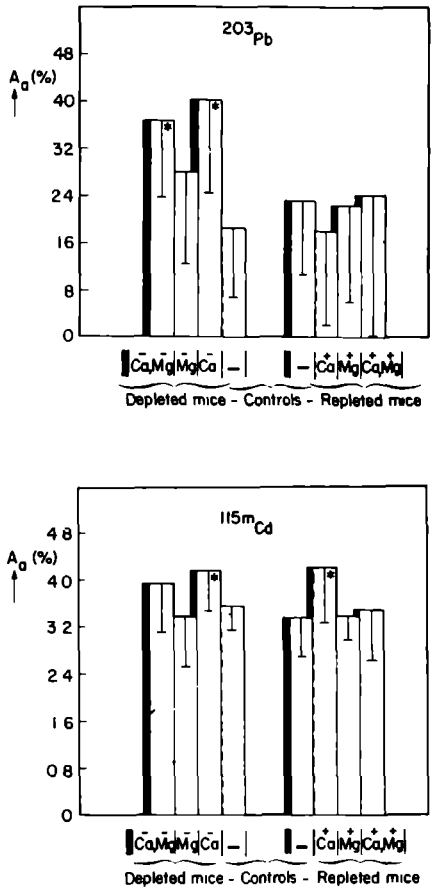


Fig. 4 and Fig. 5 Apparent absorption A_a of ^{203}Pb and ^{115m}Cd from an intubated solution as influenced by feeding of Ca- or Mg-deplete diets (exp. 2a: left) or Ca- or Mg-replete diets (exp. 2b: right) during 2 weeks prior to dosage. The mice were fasted 20 hours before dosage and 6 hours after dosage. Data are presented as mean with one standard deviation at the lower side. Statistical comparison: as in Fig. 3.

probably not absorbed because of their low solubility.

Fig. 3 shows the apparent absorption A_a of ^{115m}Cd from an intubated solution and the apparent retention R_a of intraperitoneally injected ^{115m}Cd. A_a of Cd showed a small, but not significant reduction in the presence of Ca (22%). In vitro experiments with rat duodenum showed no effect of Ca on Cd absorption [16]. In chicks, on the contrary, a competition between Ca and Cd for absorption was found [9]. The mechanism of Cd absorption in birds is probably different from that in rodents.

Fig. 2 and 3 also show that addition of Ca or Mg to the drinking water did not influence R_a of Pb or Cd. In rats it has been found that Mg addition to the food (250 mg/kg body weight/day) increased urinary Pb excretion [17]. The Mg-content of the drinking water was probably too low to produce such an effect.

Fig. 4 shows the apparent absorption A_a of ^{203}Pb from an intubated solution in mice fed a mineral-deplete or -replete diet during 2 weeks prior to dosage. A_a of Pb was strongly increased in mice fed the Ca-deplete diet A or C. It has been shown that excretion of endogenous Ca occurs mainly in the faeces [18]. Excretion of endogenous Mg, on the contrary, occurs mainly in the urine [19]. It is possible that in the mice fed a Ca-deplete diet the endogenous excretion of Ca and as a consequence the intraluminal Ca concentration was reduced. According to the results presented in Fig. 2 this could have resulted in higher absorption of Pb from the gut. In rats an increased Pb absorption has been found when a low Ca diet was fed during only 48 hours [20], which also may indicate an effect of a reduced endogenous excretion of Ca. The apparent absorption of Pb could also have been increased by a reduction of the excretion of absorbed Pb. Barton et al. [7] have found that in Ca-deprived rats the excretion of Pb is reduced, whereas the absorption is not significantly changed. Similar effects could have occurred in these mice fed a Ca-deplete diet.

Fig. 5 shows the apparent absorption A_a of ^{115m}Cd from an intubated solution in mice fed a mineral-deplete or -replete diet during 2 weeks prior to dosage. A_a of Cd was slightly increased in mice fed the Ca-deplete diet A or C and in mice fed the Ca-replete diet E, but not in mice fed the Ca,Mg-replete diet G. An increased Cd absorption has been found in Ca-depleted rats [16,21] and chicks [9]. Washko and Cousins attributed this effect to an increased activity of calcium binding protein (CaBP) [22]. The production of CaBP in the mucosal cell seems to be stimulated by a low dietary Ca intake; at the same time the diffusion of Ca through the mucosal cell membrane is facilitated [23,24]. CaBP seems to be synthesized in order to bind absorbed Ca, but it might bind Cd and Pb as well. In chicks, however, there was a lack of correlation between CaBP in the mucosa and Cd absorption. A significant role of CaBP in Cd absorption was doubted [9]. The shape of the mean retention curve of mice fed the Ca-replete diet E suggested that the

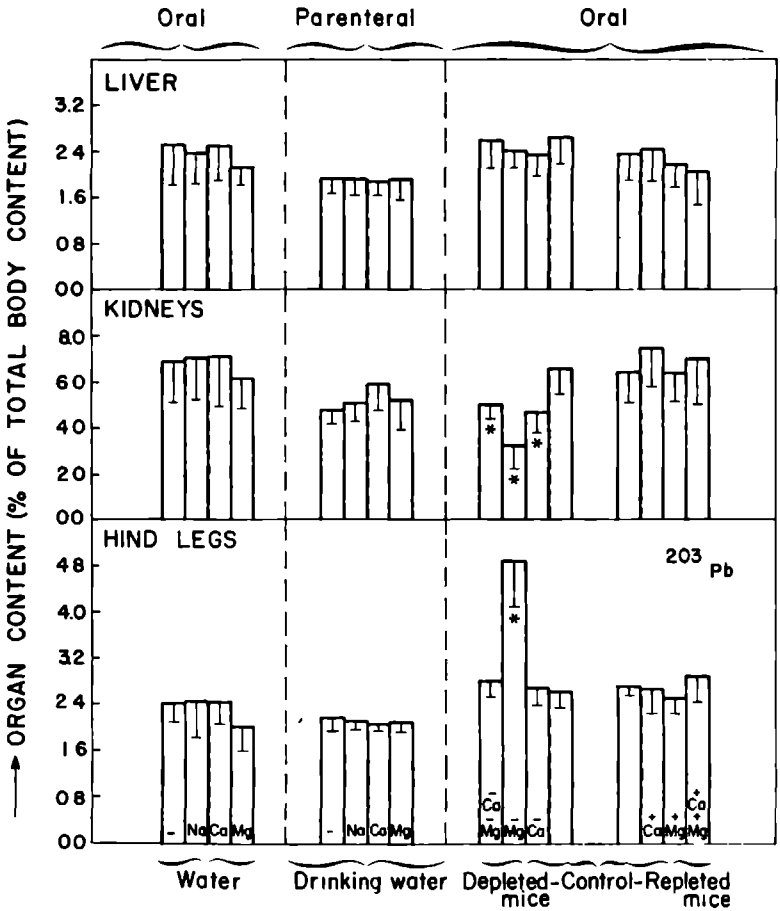


Fig. 6 ^{203}Pb content of liver, kidneys and hind legs of mice at the day of sacrifice; influence of mineral addition to the intubated solution (exp. 1a: left) or to the drinking water (exp. 1b: center) (see also Fig. 2) and of mineral depletion and repletion (exp. 2: right) (see also Fig. 4). Data are presented as mean with one standard deviation at the lower side. Statistical comparison: as in Fig. 3.

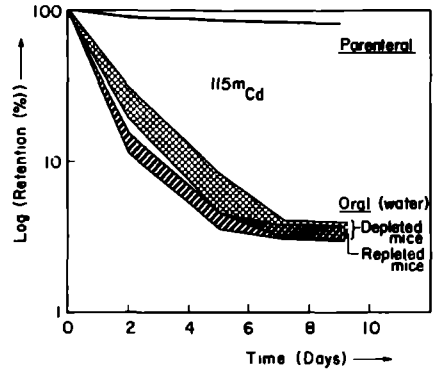
increase of A_a of Cd in these mice was caused by a reduced excretion of Cd, because a positive difference with the other curves was observed only at longer times after dosage

Feeding of the Mg-deplete diet B did not have an effect on A_a of Pb and Cd, nor did the feeding of the Mg-replete diets E and G. Cerklewski [25] found an enhanced accumulation of Pb in the liver of rats fed a Mg-deplete diet. Whether Pb absorption was increased by the absence of Mg in the food or by Mg-deficiency of the rat, was not clear

Fig. 6 shows the ^{203}Pb content of the main Pb-binding tissues, viz, liver, kidneys and bone. An interesting difference was observed in the mice fed the Mg-deplete diet B compared to the mice fed the control diet D. A strong increase in bone uptake partly at the expense of the kidneys was observed. In the mice fed the Ca,Mg-deplete diet A no increase of bone uptake was found. In contrast with the finding of Cerclewski in rats [25] the liver showed no increased Pb uptake in the mice fed the Mg-deplete diet B The uptake of Pb in the kidneys was also decreased in the mice fed the Ca- and the Ca,Mg-deplete diets A and C, without an increase in the bone. An explanation for these effects was not found.

As shown by Fig. 7, the shape of the retention curves of ^{115m}Cd, observed when the Ca,Mg-deplete diet A was fed after Cd administration, differed from the shape that was observed when the Ca,Mg-adequate diet D' was fed. The former curves indicated a retarded intestinal passage of Cd compared to the latter curves. This effect had no influence on the final body retention of Cd. It is not likely that the retained Cd was absorbed into the circulation and later on excreted, because after absorption Cd is accumulated in the organs (liver and kidneys) and its turnover is very slow [26], as is also indicated by the high retention of i.p.-injected Cd (Fig. 3) Cd might proceed through the intestine by a process of repeated binding and release, only a small fraction of it actually being absorbed [27]. Fig. 3 suggests that this process could be influenced by the presence of minerals in the food. The comparable retention curves of ^{203}Pb (Fig. 8) showed a similar effect, but here the effect was much less pronounced. Formation of complexes of Cd and Pb with Ca or Mg and phosphate from the food, which cannot be absorbed, could play a role

Table 5 shows a summary of the effects observed At the time of this study it was not known whether Ca could influence the absorption of Pb in man. Recently Blake et al. [28,29] provided evidence that in man the absorption of Pb both from water and from food is strongly increased in absence of Ca in the water or the food Their results confirm the supposed similarity between man and rodents with regard to the absorption of Pb. As a consequence, it may be concluded that the uptake of Pb from drinking water in man could be higher in soft water regions than in hard water regions because of



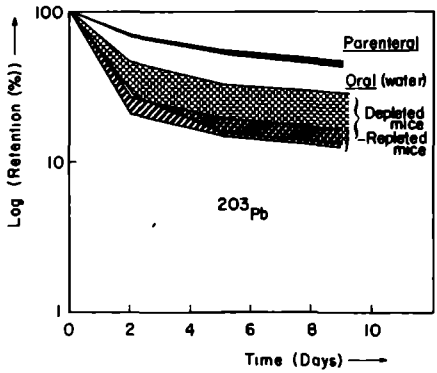


Fig. 7 and Fig. 8 Areas of comparable mean retention curves of ^{115m}Cd and ^{203}Pb, administered to mice fed Ca,Mg-adequate or Ca- or Mg-deplete diets (exp. 2a: lightly shaded area) and to mice fed Ca,Mg-adequate or Ca- or Mg-replete diets (exp. 2b: heavily shaded area) during 2 weeks prior to dosage. The former groups of mice were fed a Ca,Mg-deplete diet, the latter a Ca,Mg-adequate diet during the period of measurement after dosage.

Table 5 The influence of mineral addition to water, and of mineral depletion or repletion during 2 weeks, on ^{203}Pb and ^{115m}Cd absorption from water. [1]

^{203}Pb	Water	Depletion		Repletion	
Na [2]	-	-Ca	↑	+Ca	-
Ca [3]	↓	-Mg	-	+Mg	-
Mg [3]	-	-Ca,Mg	↑	+Ca,Mg	-
^{115m}Cd	Water	Depletion		Repletion	
Na [2]	-	-Ca	↑	+Ca	↑
Ca [3]	-	-Mg	-	+Mg	-
Mg [3]	-	-Ca,Mg	-	+Ca,Mg	-

[1] "↓" and "↑" mean a decrease or increase of apparent absorption A_a under the conditions described. [2] 50 µmole/ml or g. [3] 25 µmole/ml or g.

an increased absorption of Pb in the gastrointestinal tract in absence of Ca. The results of Meredith et al. [6], Barton et al. [7] and Hilburn et al. [8] indicate that in rats the protective effect of Ca in water is already achieved at low concentrations of Ca (2 µmole/ml [6], 10 µmole/ml [7] and 0.6 µmole/ml [8], resp.). Further addition of Ca appears to have no additional effect. It seems very important to know whether this holds true for Pb absorption in man and, if so, what would be the minimum Ca concentration in drinking water that could protect against high Pb absorption.

The effect of the Ca-status on the absorption of Pb could be particularly important in children in which Pb absorption could be increased because of their age [30]. A low dietary Ca and a low vitamin D intake have been related to high levels of Pb in blood of children [31]. This population group should be considered as a high risk group, most sensitive to increased Pb absorption from soft drinking water.

Acknowledgements

Prof. J.P.W. Houtman and dr. J.H.M. van Tongeren are acknowledged for critical review of the manuscript. Mr. C.H. Witteman is thanked for technical assistance.

References

1. Kempster, P.L. (1981) Toxicity versus essentiality - a problem in defining water quality criteria. Proc. Int. Symp. Heavy Metals in the Environment, Amsterdam, sept. 1981, 18-21.
2. Moore, M.R. (1973) Plumbosolvency of waters. Nature 243, 222-225.
3. Lyon, T.D.B. and Lenihan, J.M.A. (1977) Corrosion in solder jointed copper tubes resulting in lead contamination of drinking water. Br. Corros. J. 12, 41-45.
4. Schroeder, H.A. (1967) Cadmium, chromium and cardiovascular disease. Circulation 35, 570-582.
5. Haring, B.J.A. and Zoeteman, B.C.J. (1980) Corrosiveness of drinking water and cardiovascular disease mortality. Bull. Environm. Contam. Toxicol. 25, 658-662.
6. Meredith, P.A. , Moore, M.R. and Goldberg, A. (1977) The effect of calcium on lead absorption in rats. Biochem. J. 166, 531-537.
7. Barton, J.C. , Conrad, M.E. , Harrison, L. and Nuby, S. (1978) Effects of calcium on the absorption and retention of lead. J. Lab. Clin. Med. 91, 366-376.
8. Hilburn, M.E. , Coleman, I.P.L. and Blair, J.A. (1980) Factors influencing the transport of lead across the small intestine of the rat. Environ. Res. 23, 301-308.
9. Koo, S.L. , Fullmer, C.S. and Wasserman, R.H. (1978) Intestinal absorption and retention of 109-Cd : Effects of cholecalciferol, calcium status and other variables. J. Nutr. 108, 1812-1822.
10. Van Barneveld, A.A. and Morse, H. (1982) A new diet for trace element research with rats and mice. Z. Versuchstierk. 24, 24. IRI-Rapport 133-81-02, Delft, The Netherlands.
11. Heth, D.A. and Hoekstra, W.G. (1965) Zinc-65 absorption and turnover in rats. I. A procedure to determine zinc-65 absorption and the antagonistic effect of calcium in a practical diet. J. Nutr. 85, 367-374.
12. De Soete, D. , Gijbels, R. and Hoste, J. (1972) In : Chemical analysis, Vol. 34, Neutron activation analysis. Ed. Elving, P.J. and Kolthof, J.M. , Wiley-Interscience, London.
13. Mykkänen, H.M. and Wasserman, R.H. (1981) Gastrointestinal absorption of lead (^{203}Pb) in chicks : Influence of lead, calcium and age. J. Nutr. 111, 1757-1765.
14. Barltrop, D. and Khoo, H.E. (1975) The influence of nutritional factors on lead absorption. Postgrad. Med. J. 51, 795-800.
15. Fine, B.P. , Barth, A. , Sheffet, A. and Lavenhar, M.A. (1976) Influence of magnesium on the intestinal absorption of lead. Environ. Res. 12, 224-227.
16. Hamilton, D.L. and Smith, M.W. (1978) Inhibition of intestinal calcium

uptake by cadmium and the effect of a low calcium diet on cadmium retention. Environ. Res. 15, 175-184.

17. Singh, N.P. , Thind, I.S. , Vitale, L.F. and Pawlow, M. (1979) Intake of magnesium and toxicity of lead : an experimental model. Arch. Environ. Health 34, 168-172.
18. Roith, D. le, and Pimstone, B. (1973) The use of simultaneously administered ^{47}Ca and ^{45}Ca in the study of calcium kinetics in the rat. Horm. Metab. Res. 5, 118-123.
19. Ebel, H. and Günther, T. (1980) Magnesium metabolism: A review. J. Clin. Chem. Clin. Biochem. 18, 257-270.
20. Hart, M.H. and Smith, J.L. (1981) Effect of vitamin D and low dietary calcium on lead uptake and retention in rats. J. Nutr. 111, 694-698.
21. Washko, P.B. and Cousins, R.J. (1976) Metabolism of 109-Cd in rats fed normal and low-calcium diets. J. Toxicol. Environ. Health 1, 1055-1066.
22. Washko, P.B. and Cousins, R.J. (1977) Role of dietary calcium and calcium binding protein in cadmium toxicity in rats. J. Nutr. 107, 920-928.
23. Allen, L.H. (1982) Calcium bioavailability and absorption : a review. Amer. J. Clin. Nutr. 35, 783-808.
24. Anonymous (1978) Vitamin D, calcium-binding protein and the intestinal transport of calcium. Nutr. Rev. 36, 90-93.
25. Cerclewski, F.L. (1983) Influence of maternal magnesium deficiency on tissue lead content of rats. J. Nutr. 113, 1443-1447.
26. Cotzias, G.C. , Borg, D.C. and Selleck, B. (1961) Virtual absence of turnover in cadmium metabolism : Cd^{109} studies in the mouse. Am. J. Physiol. 201, 927-930.
27. Holt, D. and Webb, M. (1983) Intestinal and hepatic binding of cadmium in the neonatal rat. Arch. Toxicol. 52, 291-301.
28. Blake, K.C.H. , Barbezat, G.O. and Mann, M. (1983) Effect of dietary constituents on the gastrointestinal absorption of ^{203}Pb in man. Environ. Res. 30, 182-187.
28. Blake, K.C.H. and Mann, M. (1983) Effect of calcium and phosphorus on the gastrointestinal absorption of ^{203}Pb in man. Environ. Res. 30, 188-194.
30. Forbes, G.B. and Reina, J.C. (1972) Effect of age on gastrointestinal absorption (Fe, Sr, Pb) in the rat. J. Nutr. 102, 647-652.
31. Sorrell, M. , Rosen, J.F. and Roginsky, M. (1977) Interactions of lead, calcium, vitamin D, and nutrition in lead-burdened children. Arch. Environ. Hlth 32, 160-164.

5.5. PAPER X

INFLUENCE OF DIETARY CA INTAKE OF MICE ON MORTALITY FROM MG DEFICIENCY AND ON CA AND MG CONTENT OF HEART MUSCLE

A.A. Van Barneveld and C.J.A. Van den Hamer

ABSTRACT Female Swiss Random mice were fed a Ca- and Mg-deplete purified diet during 2 weeks. After this preparation period they either were fed a Mg-deplete purified diet and given drinking water with increasing Mg-concentrations (0-300 µg/ml), or were fed the same Ca- and Mg-deplete diet as before and given drinking water with increasing Ca-concentrations (0-4000 µg/ml). A high mortality was found in mice fed the Mg-deplete diet, when the drinking water contained less than 30 µg Mg/ml. No mortality was found in mice fed the Ca- and Mg-deplete diet, unless Ca was added to the drinking water; then mortality was directly related to the Ca-concentration in the water Mortality (by sudden death) in Mg-deficient mice occurred particularly shortly after introduction of Ca in the food or drinking water. The mice that survived this critical period had a better chance to stay alive in the next two weeks, probably by adaptation of the mineral metabolism. Measurement of Ca and Mg in the hearts of four selected groups of mice showed that the Ca content of the heart was strongly increased in Mg-deficient mice (high mortality groups), but reduced in Ca- and Mg-deficient mice compared to Ca- and Mg-adequate ones (low mortality groups). The Mg content of the heart was almost the same in all groups. It is suggested that a small contribution of Mg from the drinking water to the total Mg intake could be essential with respect to health, particularly when the food is low in Mg. On basis of the present knowledge Mg removal from the drinking water in case of water softening should be avoided.

Introduction

On the ground of some epidemiological studies on the possible relationship between cardiovascular diseases and water hardness attention has been focused on specific influences of Mg on cardiac physiology [1-3]. Mg seems to be particularly related to the category of arrhythmia-based sudden death [4,5].

In earlier work [6] we found a high mortality of mice fed a Mg-deficient diet during 2-5 weeks. The mice probably died as a result of acute heart failure When frightened by noise, movements or touching, they died within a few seconds. This mortality did not occur in mice fed a Mg-deficient diet that was also deficient in Ca This challenged us to suggest that the Ca/Mg-ratio in the uptake of minerals from food and drinking water could be

important rather than the Mg-deficiency. In this paper we shall extend the study of the influence of Ca on the mortality from Mg-deficiency in mice to the content of Ca and Mg in the heart muscle.

Materials and methods

Female Swiss Random mice, 4 weeks old, were obtained from The Central Institute for the Breeding of Laboratory Animals - TNO, Austerlitz, The Netherlands. A purified diet (Table 1) containing 0.4% Ca as $CaCO_3$ and 0.05% Mg as $MgSO_4.7H_2O$, a Mg-deplete variant (without Mg-sulphate) and a Ca,Mg-deplete variant (without Ca-carbonate and Mg-sulphate) were obtained from Hope Farms BV, Woerden, The Netherlands. $CaCO_3$ and $MgSO_4.7H_2O$ (pro analysi quality), used for drinking water supplements, were obtained from Merck, Darmstadt, BRD.

Table 1. Composition of IRI-CB purified diet.

Ingredient	%
Glucose	50.45
Corn starch	15.0
Casein	20.0
Sun flower seed oil	4.0
Fiber (α-cellulose)	5.0
dl Methionine	0.2
Choline chloride	0.3
Minerals [1]	3.95
Trace element mix (NETEM-80) [2]	0.1
Vitamin mix [3]	1.0

[1] Minerals (%) : $NaH_2PO_4.2H_2O$, 1.5; KCl, 0.7, $CaCO_3$, 1.0; $MgSO_4.7H_2O$, 0.5; $Na_2SiO_3.9H_2O$, 0.25. [2] Composition in mg/kg diet : Fe_2O_3, 85.9; $MnSO_4.H_2O$, 153.8; ZnO, 25.6; $CuCO_3.Cu(OH)_2$, 17.4; $NiCl_2.6H_2O$, 10.12; NaF, 5.52; $CrCl_3.6H_2O$, 10.25; $SnCl_2.2H_2O$, 3.80; KIO_3, 0.506; $Na_2MoO_4.2H_2O$, 0.504; Na_2SeO_3, 0.329; NH_4VO_3, 0.239; $Na_2HAsO_4.7H_2O$, 0.477; $CoSO_4.7H_2O$, 0.416; $Na_2B_4O_7.10H_2O$, 0.882; glucose to make 1000 mg/kg diet. [3] Composition in IU or mg/kg diet : Retinyl acetate, 16,000 IU; cholecalciferol, 1,400 IU. Menadione, 12; phylloquinone, 2; dl α-tocopheryl acetate, 85; thiamine HCl, 20; riboflavine, 12; pyridoxine HCl, 15; niacin, 40; dl calcium pantothenate, 35; vitamin B 12, 0.05; d biotin, 0.4, folic acid, 8, myo-inositol, 500 mg.

Experiment 1. Influence of dietary Ca intake on mortality from Mg-deficiency.

Twelve groups of 25 mice were housed in macrolon cages with stainless steel lids and glass drinking bottles. They were fed the Ca,Mg-deplete purified diet in combination with demineralized drinking water ad libitum during 2 weeks. After this preparation period they were placed on the following regimen (see also Table 2): 6 groups were fed the Mg-deplete purified diet ad libitum in combination with demineralized drinking water containing increasing amounts of Mg as $MgSO_4.7H_2O$ (0-300 µg Mg/ml); the other 6 groups were fed the Ca,Mg-deplete purified diet as before, ad libitum in combination with demineralized drinking water containing increasing amounts of Ca as $CaCO_3$ (0-4000 µg Ca/ml). During the following days the mortality in the groups was registered and on the 17th day the remaining mice were sacrificed.

Table 2 Mineral composition of food and drinking water in 12 test groups.

Group	Food	Water Mg (µg/ml)	Treatm.	Group	Food	Water Ca (µg/ml)	Treatm.
1	+Ca,-Mg	0	Mg-depl.	7	-Ca,-Mg	0	Ca,Mg-depl
2	"	3		8	"	200	
3	"	10		9	"	500	
4	"	30	↓	10	"	1000	↓
5	"	100		11	"	2000	
6	"	300	Adeq.	12	"	4000	Mg-depl.

Experiment 2. Influence of dietary Ca intake on Ca and Mg content of the heart

Experiment 1 was partly repeated with four groups of 25 mice, representing the four extreme groups 1, 6, 7 and 12 (Table 2). On the 17th day of the second diet period none of the mice in groups 1, 6 and 7, and 9 mice in group 12 had died. The remaining mice were sacrificed by bleeding. From 10 mice of each group the heart was dissected, freeze-dried and destructed in nitric acid and hydrogen peroxide. The contents of Ca and Mg in the hearts were determined by Induced Coupled Plasma Spectrometry (Instruments SA, JY 38 PI).

Results and discussion

During the first 2 weeks all groups were fed a diet low in both Ca and Mg; no adverse effects of this treatment were observed. Shortly after Ca-repletion of the diet by addition of Ca, either to the food (groups 1-6) or to the drinking water (groups 8-12) a high mortality was found in groups 1-3 and in groups 8-12, which in the latter groups was directly related to the Ca-concentration in the water (Fig. 1-2) Even at the lowest Ca concentration in the drinking water (group 8· 200 µg Ca/ml) 7 out of 25 mice died within 17 days When demineralized water was given (group 7) only 1 out of 25 mice died in the same period In mice fed the Mg-deplete diet (groups 1-6) the mortality was obviously reduced by addition of Mg to the drinking water. Total reduction of the mortality was achieved at a concentration of 30 µg Mg/ml (group 4).

Table 3 shows the results of Ca and Mg analyses in heart muscle for four extreme groups of mice, viz, group 1, 6, 7 and 12 (see Table 2). The Mg content of the heart appeared to be almost independent of the dietary Mg intake, which is in agreement with results in rabbits [7] and rats [8-10] The Ca-content of the heart, however, was strongly increased in the Mg-deficient

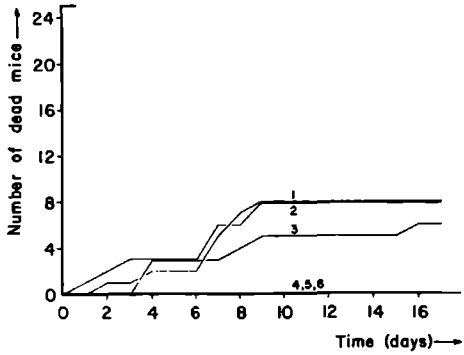


Fig. 1 Cumulative mortality in mice fed a Mg-deplete diet in combination with demineralized drinking water containing increasing amounts of Mg (group 1-6: 0, 3, 10, 30, 100, and 300 µg Mg/ml, resp.). At the start of the experiment each group contained 25 mice.

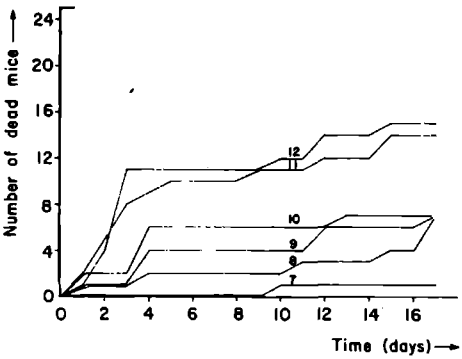


Fig. 2 Cumulative mortality in mice fed a Ca,Mg-deplete diet in combination with demineralized drinking water containing increasing amounts of Ca (groups 7-12: 0, 200, 500, 1000, 2000, and 4000 µg Ca/ml, resp.). At the start of the experiment each group contained 25 mice.

Table 3 Ca and Mg content of the heart of the mice in groups 1 (Mg-deplete), 6 (adequate), 7 (Ca,Mg-deplete) and 12 (Mg-deplete). [1]

Group	Treatment	Ca (µg/g) [2]	Mg (µg/g) [2]
1	Mg-depletion	350± 80 $^{\alpha}$	880± 30 $^{\epsilon}$
6	Adequate	110± 40 $^{\beta}$	910± 40 $^{\delta}$
7	Ca,Mg-depletion	70± 30 $^{\gamma}$	840± 60 $^{\epsilon\partial}$
12	Mg-depletion	350±100 $^{\alpha}$	800± 50 $^{\partial}$

[1] Means with common superscripts (α, β, γ, δ, ϵ, ∂) are not significantly different by Student's t-test ($P<0.05$). [2] dry weight (n=10).

groups 1 and 12, those which also exhibited the highest mortality. Obviously, this Ca accumulation in the heart muscle could be inhibited not only by Mg suppletion in the drinking water, but also by feeding a Ca-deplete diet. In the latter case even a reduction of the Ca content of the heart was observed.

In Mg-deficiency accumulation of Ca in tissues, even to a level that causes histochemical and histological changes (calcification), seems to be quite common. Calcification due to Mg-deficiency has been found particularly in kidneys and to a lesser extent also in heart and aorta [8-15]. As far as known to the authors, the effect of Ca-depletion on calcification due to Mg-deficiency has not been investigated before.

In experiment 2 no mortality was found in group 1 and a lower mortality in group 12 compared to experiment 1. The reason for this difference is not understood. It could be that the $CaCO_3$ that was used to prepare the Mg-deplete diet in experiment 2 contained a trace of Mg. Analysis of the Ca,Mg-deplete diet and the Mg-deplete diet used in this experiment showed that the Mg content was 16±1 µg/g and 72±1 µg/g, respectively. Although 72 µg Mg/g food is still very low, such differences obviously could strongly influence the results with respect to mortality.

Table 4 Ranges of Ca and Mg concentrations in soft, normal and hard drinking water in The Netherlands [16].

	Ca (µg/ml)	Mg (µg/ml)
Soft water	<45	1-5
Normal water	45-85	5-15
Hard water	>85	6-40

It is noteworthy that the concentration of Mg in the drinking water, needed to reduce mortality from Mg deficiency in mice to zero, was 30 µg/ml, which is in the range of values occurring in practical hard drinking water in The Netherlands (Table 4). Therefore, Mg in drinking water, though normally being quantitatively negligible compared to food Mg, may be qualitatively important for protection against Mg-deficiency, when the diet is very low in Mg.

In man a severe deficiency of Mg because of nutritional reasons is not very likely [17,18]. A marginal Mg-status caused by a low or sub-adequate Mg intake from the food may, however, occur more generally [2]. In such case, a small contribution of Mg from the drinking water could be essential to avoid Mg-deficiency and possible cardiac malfunctioning resulting from this. Therefore, in softening of drinking water removal of Mg should be avoided. As about 85% of water hardness is caused by Ca [16], selective elimination of Ca will produce the desired softness without affecting the Mg-intake.

Acknowledgements

Prof. J.P.W. Houtman and dr. J.H.M. Van Tongeren are acknowledged for critical review of the manuscript. Ing. A. Klok, Mr. P.A. Jessen and Mr. C.A. Witteman are acknowledged for analysis of Ca and Mg in heart muscle and food by ICPS.

References

1. Neri, L.C. & Johansen, H.L. (1978). Water hardness and cardiovascular mortality. Ann. N. Y. Acad. Sci. 304, 203-219.
2. Karppanen, H. (1981) Epidemiological studies on the relationship between magnesium intake and cardiovascular diseases. Artery 9, 190-199
3. Manthey et al. (1981) Magnesium and trace metals: risk factors for coronary heart disease? Circulation 64, 722-729
4. Dyckner, T. & Wester, P.-O. (1982) Magnesium in cardiology Acta Med Scand. 661, 27-31
5. Ebel, H. & Günther, T (1983) Role of magnesium in cardiac disease J Clin. Chem. Clin. Biochem 21, 249-265.
6. Van Barneveld, A.A., Van den Hamer, C.J.A. & Houtman, J.P.W. (1982) Drinking water hardness, trace elements and cardiovascular diseases. Influence of Ca and Mg on metabolism of Cd, Co, Cu, Zn and Se in mice. Tr Subst Environm Hlth 16, 196-204.
7. Aikawa, J.K., Reardon, J.Z. & Harms, D.R. (1962) Effect of a magnesium-deficient diet on magnesium metabolism in rabbits a study with Mg^{28} J. Nutr 76, 90-93.
8. Britton, W.M. & Stokstad, E.L.R. (1970) Aorta and other soft tissue calcification in the magnesium-deficient rat J Nutr 100, 1501-1506

9. Heroux, O., Peter, D. & Heggtveit, A. (1977) Long-term effect of suboptimal dietary magnesium on magnesium and calcium contents of organs, on cold tolerance and on lifespan, and its pathological consequences in rats. J. Nutr. 107, 1640-1652.
10. Fischer, P.W., Giroux, A., L'Abbé, M. & Nera, E.A. (1981) The effects of moderate magnesium deficiency in the rat. Nutr. Rep. Int. 24, 993-1000.
11. Heggtveit, H.A., Herman, L. & Mishra, R.K. (1964) Cardiac necrosis and calcification in experimental magnesium deficiency. A light and electron microscopic study. Am. J. Pathol. 45, 757-782.
12. Goldsmith, L.A. (1967) Relative magnesium deficiency in the rat. J. Nutr. 93, 87-102.
13. Goulding, A. & Malthus, R.S. (1969) Effect of dietary magnesium on the development of nephrocalcinosis in rats. J. Nutr. 97, 353-358.
14. Ryan, M.P. & Ryan, M.F. (1977) Muscle calcium accumulation during magnesium deficiency in the rat. Biochem. Soc. Trans. 5, 1744.
15. Harwood, E.J. (1982) The influence of dietary magnesium on reduction of nephrocalcinosis in rats fed purified diet. Lab. Animals 16, 314-318.
16. Kalkman, C. (1979) Magnesium, hart/vaatziekten en de waterfactor; een literatuuroverzicht. Universiteit van Amsterdam, Faculteit der Geneeskunde, Huisdrukkerij Universiteit van Amsterdam T-7430.
17. Flink, E.B. (1981) Magnesium deficiency. Etiology and clinical spectrum. Acta Med. Scand., suppl. 647, 125-138.
18. Ebel, H. & Günther, T. (1980) Magnesium metabolism: A review. J. Clin. Chem. Clin. Biochem. 18, 257-270.

5.6. Summary.

The influence of Ca and Mg on the absorption and retention of 7 trace elements (Zn, Cu, Mn, Co, Pb, Cd and Se, the last as SeO_3^{2-}) in mice was investigated under three different conditions.

Firstly, direct competitive interaction was investigated by intragastric intubation of the TE's in water, supplemented with Ca or Mg (25 µmole/ml). Secondly, indirect interaction - mediated by food components - was investigated by administration of the TE's mixed with portions of (purified) food, supplemented with Ca or Mg (25 µmole/g). Thirdly, the effect of changing the Ca or Mg status of mice on the absorption and retention of the TE's was investigated by feeding of Ca- or Mg-deplete diets (adequate minus 0.4% Ca or 0.05% Mg, resp.) or Ca- or Mg-replete diets (adequate plus 1.0% Ca or 0.125% Mg, resp.) during at least 2 weeks followed by intragastric intubation of the TE's in water.

All these investigations were carried out with the aid of radiotracers of the TE's. Retention curves of the administered TE's were measured, from which the apparent absorption A_a (after oral administration) and the apparent retention R_a (after parenteral administration) were calculated (for the physiological meaning of A_a and R_a, see Section 3.5). A_a was used to describe the effect of Ca and Mg in water and food and of the Ca- or Mg-status on the absorption of TE's. R_a was used to describe the effect of Ca and Mg in drinking water on the body retention of TE's. The results are shown in Table 1 and 2.

It should be kept in mind that the value of A_a results from both true absorption (A_t) and endogenous excretion (S_{en}) (see Section 3.5). It may be safely assumed, however, that the effects caused by Ca or Mg in the first group of experiments presented in Table 1 (TE absorption from water or food, influenced by their Ca or Mg content) were absorption effects, because the Ca and Mg additions were negligible compared to the plasma pool of Ca and Mg and will probably not have influenced TE excretion. It is therefore allowed to speak of lower and higher absorption without reference to the "apparent" character of the effect.

In the second group of experiments, presented in Table 2 (TE absorption from water as influenced by the Ca- or Mg-status), the influence of the status on TE excretion may have had a significant impact on the measured A_a. Here both absorption and excretion effects should be considered.

Table 1 Influence of food compared to water and of addition of Ca or Mg to both on the apparent absorption (A_a), and influence of addition of Ca or Mg to the drinking water on the apparent retention (R_a) of TE s. [1]

TE	A_a (food) [2] (adequate)	A_a (water) +Ca[3]	A_a (water) +Mg[3]	A_a (food) +Ca[4]	A_a (food) +Mg[4]	R_a +Ca[5]	R_a +Mg[5]	Section
Zn	↓	↓	-	-	-	-	-	5.2
Cu	↓	-	-	-	-	-	-	5.2
Mn	↓	↓	↓	↓	↓	-	-	5.3
Co	↓	↓	↓	-	-	-	-	5.2
Pb	↓ [6]	↓	-	[7]	[7]	-	-	5.4
Cd	↓	-	-	↓	↓	-	-	5.2/4
Se	↑	-	-	-	-	-	-	5.2

[1] "↓", "↑" and "-" mean a decrease and increase of the A_a and no effect, resp., under the condition indicated. [2] Effect of standard food (purified diet) compared to water (demineralized). [3] 25 µmole/ml, in water (demineralized). [4] 25 µmole/g, in food (Ca,Mg-deplete). [5] 25 µmole/ml, in the drinking water. [6] Unpublished results. [7] Data not available.

As shown in Table 1, Ca reduced the absorption of Zn, Mn, Co and Pb from water and the absorption of Mn and Cd from food. Mg reduced the absorption of Mn and Co from water and the absorption of Mn and Cd from food. The effects of Ca and Mg on the absorption of Zn and Cd were small, those on the absorption of Mn and Co large. In view of the 5-fold excess of Ca and the 50-fold excess of Mg in the experimental water as compared to practical hard water it does not appear likely that in mice drinking water hardness will influence the absorption of Zn, Cu, Cd and Se. The absorption of Mn, Co and Pb from drinking water might be reduced in mice due to the hardness of the water.

Food, adequate in Ca and Mg, reduced the absorption of all cationic TE's compared to demineralized water. This could partly be caused by its mineral and TE content, partly to the binding of the TE's to indigested food components. Se showed an opposite effect. The retention of intraperitoneally injected TE's was not influenced by the addition of Ca or Mg to the drinking water.

The effects of Ca and Mg on the absorption of Mn, Co, Pb and Cd has been extensively discussed in Section 5.3 and 5.4. The effect of Ca on Zn absorption is discussed in a Note added to the present Section.

Table 2 Influence of the Ca- or Mg-status on the apparent absorption A_a of TE's from water. [1]

TE	Status [2]						
	-Ca	-Mg	-Ca,-Mg	+Ca	+Mg	+Ca,+Mg	Section
Zn	-	-	-	-	-	-	5.2
Cu	-	-	-	-	-	-	5.2
Mn	↑	-	↑	-	-	-	5.3
Co	↓	↓	↓	-	-	-	5.2
Pb	↑	-	↑	-	-	-	5.4
Cd	↑	-	-	↑	-	-	5.4
Se	-	-	-	↑	-	-	5.2

[1] "↓", "↑" and "-" mean a decrease and increase of the A_a and no effect, resp, under the condition indicated. [2] The Ca- or Mg-status was influenced by feeding of a Ca- or Mg-deplete diet (= adequate minus 100 µmole Ca/g or minus 20 µmole Mg/g or minus both) or a Ca- or Mg-replete diet (= adequate plus 250 µmole Ca/g or plus 50 µmole Mg/g or plus both) during two weeks prior to experimentation.

Ca-depleted mice retained more Mn, Pb and Cd and less Co than normal mice. Mg-depleted mice only retained less Co than normal mice. Concerning Mn and Pb the absorption could have been increased because of a lower luminal content of endogenous Ca in the Ca-depleted mice. For the effects on Co absorption no explanation was found. Absorption of Pb and Cd could have been increased as a result of homeostatic adaptation of the Ca absorption. The excretion of Pb or Cd could have been reduced as well.

The main results of the experiments have been presented on the Int. Symp. "Health effects and interactions of essential and toxic elements", June 1983, Lund, Sweden. The text of this presentation has been taken up in Appendix III.

An additional but important result of the study with Ca- or Mg-deplete diets was the mortality found in mice fed a Mg-deplete diet during several weeks. This mortality could be eliminated not only by Mg suppletion (addition of Mg to the drinking water: 1.25 µmole Mg/ml), but also by Ca-depletion simultaneous with Mg-depletion. Addition of Ca to the drinking water (upto 100 µmole/ml) accentuated Mg-deficiency. The dietary Mg intake did not influence the Mg content of the heart muscle. The Ca content of the heart muscle was strongly increased in Mg-deficient mice, but not in Ca-deprived Mg-deficient mice.

Note

The absorption of Zn from water was found to be slightly reduced by Ca (Section 5.2), which confirms the results of Adham and Song [1] They found in rats a reduction of Zn absorption from an intubated solution by 25 µmole Ca/ml, but not by 12.5 µmole Ca/ml. Mg did not exhibit any effect in the experiments with Zn. The absence of an effect of Ca on Zn absorption from (purified) food contrasted with results of Heth et al [2] and of Cabell and Earle [3] who found that Ca reduced Zn absorption from purified diets, based on casein and soy protein, resp. Both investigators, however, used much larger additions of Ca in their experiments (125-300 µmole/g) as compared to the present experiments (25 µmole/g). Furthermore the diet of Cabell and Earle may have contained some phytate in the soy protein. Huber and Gershoff [4] found in rats fed a casein-based purified diet a reducing effect of 325 µmole Ca/g on Zn absorption only in zinc-deficient, but not in zinc-adequate mice. Obviously the Zn-status also plays a role.

Literature cited.

1. Adham, N.F. and Song, M.K (1980) Effect of calcium and copper on zinc absorption in the rat. Nutr. Metab. 24, 281-290.
2. Heth, D.A., Becker, W.M. & Hoekstra, W.G. (1966) Effect of dietary calcium, phosphorus and zinc on zinc-65 absorption and turnover in rats fed semipurified diets. J. Nutr 88, 331-337.
3. Cabell, C.A. & Earle, I.P. (1965) Additive effect of calcium and phosphorus on utilization of dietary zinc. J Animal Sci. 24, 800-804
4. Huber, A.M. & Gershoff, S.N. (1970) Effects of dietary zinc and calcium on the retention and distribution of zinc in rats fed semipurified diets. J. Nutr. 100, 949-954

5.7. GENERAL CONCLUSIONS

When Ca or Mg are mentioned as such, raised but not excessive concentrations of minerals (25 µmole/ml or 25 µmole/g) compared to hard water are meant.

1. The absorption of (cationic) trace elements is generally higher from water than from food.
2. Both Ca and Mg reduce the absorption of Mn and Co from water and the absorption of Mn from food.
3. Ca reduces the absorption of Zn and Pb from water.
4. The retention of Zn, Cu, Mn, Co, Pb, Cd and Se is not influenced by Ca or Mg in the drinking water.
5. The absorption of Mn, Pb and Cd may be increased by a low dietary Ca intake.
6. The absorption of Co may be reduced by a low dietary Ca or Mg intake.
7. The excretion of ^{6}b may be reduced by a low dietary Ca intake.
8. The excretion of Cd may be reduced by a high dietary Ca intake.
9. Mortality from Mg-deficiency is eliminated both by Mg in the drinking water (1.25 µmole/ml) and by Ca-deprivation simultaneous with Mg-depletion.
10. With regard to mortality Ca accentuates the effects of Mg-deficiency.

These conclusions are only valid for mice under the experimental conditions described. The relative importance of the findings for man are discussed in Chapter 6.

CHAPTER 6

EVALUATION OF SOME RESULTS WITH RESPECT TO PUBLIC HEALTH

6.1. The influence of drinking water hardness on trace element uptake in man.

It should be questioned whether the observed effects of Ca and Mg on the absorption and retention of trace elements (abbr. TE's) in mice (see paragraph 5.7) are relevant to TE uptake in man. Although many similarities between man and mice exist with respect to TE metabolism, it is not certain that a similarity exists in every aspect. Therefore, studies in mice can only be indicative for the existence of possible effects in man, but these effects should be confirmed by comparable human studies.

Several studies have been carried out in man concerning the influence of Ca or Mg on TE balance. The results are shown in Table 1 and are characterized by absence of any effect of Ca.

Table 1 Results of balance studies of TE's in man.

Mineral	Dose (g)	Form	TE Balance	Effect	Subject	Ref.
Ca	1.3	Tablet	Zn, Cu, Mn	No	Girls	[1]
Ca	3.0	Tablet	Zn, Cu, Co, Cd, Se	No	Males	[2]
Ca	1.7-2.1	Dairy	idem	No	Females	[2]
Ca	2.4	Diss.	Zn, Cu, Mn, Se, Fe	No	Males	[3]
Ca	1.5	Tablet	Zn, Cu	No	Females	[4]
Ca	2.4	Diss.	Zn, Cu, Fe	No	Males	[5]
Ca	0.8	Tablet	Zn, Cu, Mn, Cd	No	Males	[6]
Ca	0.8	Milk	idem	No	Males	[6]
Ca	0.8-2.0	Tablet	Zn	No	Males	[7]
Mg	$25\text{-}75\cdot10^{-3}$	?	Zn	No	Adults	[8]

Only very few radiotracer studies have been carried out in man to investigate the effect of Ca on TE absorption and retention. These are listed in Table 2.

Table 2 Results of radiotracer studies on TE absorption as influenced by Ca supplementation. [1]

Radioisotope	Carrier	Source of Ca	Effect	Subject	Ref.
^{65}Zn	Water	Tablet	No	Adults	[9]
^{65}Zn	Water	Tablet	No	Adults	[7]
^{65}Zn	Bread	Milk	↑	Adults	[10]
^{65}Zn	Soybean	Milk	↓	Adults	[11]
^{203}Pb	Water	$CaCO_3$	↓	Adults	[12,13]
^{203}Pb	Food	$CaCO_3$	↓	Adults	[12]
^{203}Pb	Milk	Milk	↓	Adults	[13]

[1] "↓" and "↑" mean a decrease or increase, resp., of the absorption of the TE in question by Ca supplementation.

On basis of the available literature and of the information obtained during the present study it may be assumed that drinking water hardness will not influence the balance of Zn, Cu and Se. Water hardness could reduce the absorption of Mn from drinking water and possibly also from food prepared with drinking water. However, homeostatic regulation of Mn is very efficient, so an increase of Mn absorption will probably result in an equivalent increase of Mn excretion with the bile.

Concerning Co no human data are available. The present study indicates that an increased absorption of Co from drinking water due to the softness of the water is partly compensated by an increased urinary excretion of Co. The net retention of Co could be slightly increased when soft water is used. However, this will hardly contribute to the daily intake of Co from the food.

From epidemiological investigations it is known, that in Itai-itai disease Cd toxicity is strongly accentuated by a low dietary Ca intake. Animal studies indicate, however, that the reason for increased toxic effects of Cd (particularly on bone structure) is not an increased absorption or retention of Cd, but rather an increased effect of Cd on bone mineralization as a result of the poor Ca-status. A hypertensive effect of Cd is not found in the Itai-itai disease. It is suggested that hypertension only occurs at chronic low level intake of Cd, which does not cause symptoms of severe Cd intoxication. Nevertheless, this subject is controversial, so it would be speculative to say that the small effects of Ca and of the Ca-status on Cd absorption can be related to hypertension or other causes of cardiovascular disease. If there is any relation between water hardness and cardiovascular disease mediated by Cd, then the release of Cd from water pipes probably would play a main role.

Water hardness may play an important role in the daily uptake of Pb. The contribution of drinking water to Pb uptake might be relatively high, because absorption of Pb from water is much higher than that from food [12, 14] and because Pb extracted from the piping may be an important source of Pb contamination of drinking water. It should be realized that also some of the Pb entering the body as aerosols will eventually find its way through the bronchi to the esophagus and may be swallowed with drinking water, thus contributing to Pb absorption from the gut.

The present study showed in mice a lower absorption of Pb from Ca-containing water (25 µmole/ml) than from demineralized water. The studies of Blake and Mann [12,13] show a similar result for man. They found that the fraction of ^{203}Pb retained from demineralized water at 96 h after dosage was reduced from 70% to 17% by addition of 0.07 g Ca and to 2% by addition of 0.7 g Ca in 150 ml water [13] (11.7 µmole Ca/ml and 117 µmole Ca/ml, resp.). Omitting minerals from the food increased ^{203}Pb retention at 96 h from 4% to 35% [12]. These results indicate that Ca may have a very important protective function against Pb absorption.

Blake and Mann suggest that addition of milk to a Ca-poor diet could protect against Pb-uptake, as they found that Pb absorption from milk (16%) was much lower than that from demineralized water (70%) [13]. Such addition could reduce the absorption of Pb from food prepared with Pb containing drinking water, but probably not from drinking water consumed as such. It cannot be excluded that milk promotes Pb absorption from food [15]. Addition of milk to the diet might therefore have an opposite (absorption stimulating) effect on Pb uptake.

As Pb may already be toxic at very low intake, particularly in children, increased Pb absorption due to absence of Ca in the drinking water should be avoided. Central softening of water should maintain a minimal level of Ca in the drinking water. This minimal level protecting against increased Pb uptake from drinking water should be determined by further investigation.

6.2. Significance of Mg as the "water factor".

One of the factors that have been mentioned as the "water factor" causing the negative correlation between drinking water hardness and mortality from cardiovascular diseases (CVD) is the Mg content of the drinking water [16] In his review on the role of Mg in drinking water with respect to CVD, Kalkman [17] concluded that (1) the Mg-supply of some population groups could be insufficient, (2) Mg in drinking water could significantly contribute to the daily Mg-uptake, (3) soft water could promote CVD because of its low Mg content.

Symptomatic Mg-deficiency seems to occur only under abnormal health conditions, such as malabsorption, renal disease, hormonal disturbances and severe malnutrition, e.g., due to starvation or alcoholism [18]. When the dietary intake of Mg is sub-adequate only, the body reacts on the low Mg intake by reducing urinary Mg excretion to prevent depletion. Furthermore, the bone could serve as a depot of available Mg that may be mobilized to keep the Mg content of the soft tissues at a constant level. Therefore, the body has a large capacity to cope with low dietary Mg. On the other hand, mild Mg-deficiency in rats leads to Ca accumulation in soft tissues [19], probably by bone demineralization necessary for Mg mobilization from the bone and by a reduced urinary excretion of Ca. Even when no histological changes are observed, Ca accumulation could lead to an increase of the Ca/Mg-ratio in the cell, which may influence electric stimulus conduction. In a more advanced stage this could cause the increased excitability, tremors, convulsions, spasms and cardiac arrhythmias observed in severe Mg deficiency [18]. Mild Mg deficiency might not be symptomatic, but an increased Ca/mg-ratio in the heart could increase the risk of cardiac failure.

The present results (section 5.6) show that in mice Mg in drinking water may reduce mortality caused by Mg-deficiency. Though the food given to the mice was extremely low in Mg, the concentration of Mg in the drinking water required to eliminate mortality (30 µg/ml) was in the range of concentrations occurring in hard waters in The Netherlands (6-40 µg/ml) [17]. This suggests that the Mg content in hard drinking water might be sufficient to improve a sub-optimal Mg-status caused by a diet with a Mg content below adequate.

Several epidemiological studies indicate that the dietary intake of Mg may be sub-adequate in many industrialized countries [20, 21]. This may perhaps not lead to overt Mg-deficiency symptoms, but in a large population it could raise the number of deaths from CVD. A small contribution of drinking water to the total Mg intake could protect against CVD, particularly because Mg in drinking water could be absorbed more readily than Mg in food [22]. As it cannot be assured that the protective effect of Mg in hard drinking water against CVD is negligible, it seems prudent to conserve Mg in the drinking water when softening of water is desired.

Literature cited.

1. Price, N.O. & Bunce, G.E. (1972) Effect of nitrogen and calcium on balance of copper, manganese, and zinc in preadolescent girls. Nutr. Rep. Int. 5, 275-280.
2. Wester, P.O. (1974) Trace element balances in relation to variations in calcium intake. Atherosclerosis 20, 207-215.
3. Greger, J.L., Snedeker, S.M. & Smith, S.A. (19??) Effect of dietary calcium and phosphorus levels on mineral utilization of adult males.
4. Johnson, N.E., Greger, J.L., Kusserow, K., Seyedabi, F. & Smith, E.L. (1981) Zinc, copper, and magnesium in serum of women given a calcium supplement. Proc. 3rd Int. Symp. TEMA-4, M. Kirchgessner, ed., pp. 237-240.
5. Snedeker, S.M., Smith, S.A. & Greger, J.L. (1982) Effect of dietary calcium and phosphorus levels on the utilization of iron, copper, and zinc by adult males. J. Nutr. 112, 136-143.
6. Spencer, H., Asmussen, C.R., Holtzman, R.B. & Kramer, L. (1979) Metabolic balances of cadmium, copper, manganese, and zinc in man. Am. J. Clin. Nutr. 32, 1867-1875.
7. Spencer, H., Kramer, L. & Osis, D. (1983) Zinc balances in humans during different intakes of calcium and phosphorus. In: Nutritional bioavailability of zinc, pp. 223-232, Inglett, G.E., ed., American Chemical Society, Washington D.C., USA.
8. Geders, J.M., Brown, T.D. & Crews, M.G. (1981) Effect of magnesium on zinc absorption. Fed. Proc. 41, 938 (abstract 4019).
9. Spencer, H., Vankinscott, V., Lewin, I. & Samachson, J. (1965) Zinc-65 metabolism during low and high calcium intake in man. J. Nutr. 86, 169-177.
10. Sandström, B., Arvidsson, B., Cederblad, Å & Björn-Rasmussen, E. (1980) Zinc absorption from composite meals. I. The significance of wheat extraction rate, zinc, calcium, and protein content in meals based on bread. Am. J. Clin. Nutr. 33, 739-745.
11. Sandström, B. & Cederblad, Å (1980) Zinc absorption from composite meals. II. Influence of the main protein source. Am. J. Clin. Nutr. 33, 1778-1783.
12. Blake, K.C.H., Barbezat, G.O. & Mann, M. (1983) Effect of dietary constituents on the gastrointestinal absorption of ^{203}Pb in man. Environ. Res. 30, 182-197.
13. Blake, K.C.H. & Mann, M. (1983) Effect of calcium and phosphorus on the gastrointestinal absorption of ^{203}Pb in man. Environ. Res. 30, 188-194.
14. Rabinowitz, M.B., Kopple, J.D. & Wetherill, G.W. (1980) Effect of food intake and fasting on gastrointestinal lead absorption in humans. Am. J. Clin. Nutr. 33, 1784-1788.
15. Kostial, K. & Kello, D. (1979) Bioavailability of lead in rats fed "huma"

diets. Bull. Environm. Contam. Toxicol. 21, 312-314.

16. Neri, L.C. & Johansen, H.L. (1979) Water hardness and cardiovascular mortality. Ann. N.Y. Acad. Sci. 304, 203-219.
17. Kalkman, C. (1979) Magnesium, hart/vaatziekten en de waterfactor; een literatuuroverzicht. Universiteit van Amsterdam, Faculteit der Geneeskunde, Huisdrukkerij Universiteit van Amsterdam T-7430.
18. Flink, E.B. (1981) Magnesium deficiency. Etiology and clinical spectrum. Acta Med. Scand. suppl. 647, 125-138.
19. Fischer, P.W., Giroux, A., L'Abbé, M. & Nera, E.A. (1981) The effects of moderate magnesium deficiency in the rat. Nutr. Rep. Int. 24, 993-1000.
20. Marier, J.R., Neri, L.C. & Anderson, T.W. (1979) Water hardness, human health, and the importance of Mg. National Research Council Canada, NRCC No. 17581.
21. Karppanen, H. (1981) Epidemiological studies on the relationship between magnesium intake and cardiovascular diseases. Artery 9, 190-199.
22. Löwik, M.R.H., Groot, E.H. & Binnerts, W.T. (1982) Magnesium and public health: The impact of drinking water. Tr. Subst. Environm. Hlth 16, 189-195.

SUMMARY

In the last decade the interest of physicians, biochemists and toxicologists for the biological role of trace elements has strongly increased. This can be seen from the increasing number of international conferences on trace element metabolism, the edition of new scientific journals on the subject and the increasing emphasis on trace elements in the already existing literature. After a first surge of mainly qualitative research at present attention is focused particularly on quantitative aspects. Deficiencies, both clinical and sub-clinical, of elements known to be essential (Zn, Cu, Se, etc.) and environmental contamination with elements known to be toxic (Cd, Pb, As, etc.) are subject of intensive research.

The terms "essential" and "toxic" appear to be relative: all "essential" elements are, if present in large quantities, toxic and many "toxic" elements ultimately appear to be essential. The significance of a trace element for an organism is therefore determined by, among others, the quantity taken up in the body. This depends on the one hand on the supply and bioavailability of trace elements, on the other hand on the capacity of the organism to take up and retain these trace elements. Disturbances of the trace element balance can generally be attributed to these aspects.

The present study focuses on the absorption and retention of radiotraced trace elements in mice and particularly on the conditions (among others, the presence of Ca and Mg) that may influence these processes. The first part of the study has been carried out to gain insight in the role of such conditions in metabolic experiments: to recognize these as factors influencing the results, to be able to design experiments appropiately and with high reproducibility and to facilitate intercomparison between experiments performed under different conditions.

Basic to trace element research is the use of a suitable laboratory animal diet of constant composition and containing adequate but not excessive levels of minerals and trace elements and a minimal level of trace element complexing agents. Such a diet (IRI-CB purified diet) was composed and tested (4.2) and used throughout the study.

The influence of this diet on trace element absorption was investigated in mice for seven elements, viz., Zn, Cu, Mn, Co, Pb, Cd and Se (4.3). For most elements changes in absorption were found after feeding the diet during two weeks. Obviously the metabolism was adapted to the lower trace element intake from the purified diet. Particularly for the pilot element Zn two weeks appeared to be a suitable adaptation period. This period was applied to all elements and in all studies.

Using Zn as pilot element various methods of trace element administration were tested (4.4). The influence of fasting before, during and after

trace element administration was also investigated. Administration of Zn in drinking water appeared to be equivalent to intragastric intubation of a Zn solution. Administration of Zn in food, however, strongly reduced its absorption. A similar effect occurred when food was consumed immediately before, during or after administration of Zn in solution. This food effect demands standardization of feeding conditions during absorption experiments.

The absorption and retention of radiotracers of Zn, Cu, Se and Pb generally appeared to depend on the concentration of these elements in the administered dose (4.5). This concentration should therefore be chosen deliberately. The obtained results offered insight in the various mechanisms of trace element homeostasis in mice.

The intestinal passage of Cu and Zn was investigated in both mice and rats (4.6). Insight was gained in the element-specific character of the speed of intestinal passage and of the degree of absorption and in the effect of food consumption after trace element administration on these factors.

Finally the body distributions of intravenously and intraperitoneally administered Zn were compared and were found to be almost identical (4.7).

With the gained knowledge of and skill in performing absorption and retention experiments it was possible to start the second part of the study, viz., the investigation of the influence of Ca and Mg on the uptake of trace elements from drinking water and food. This influence deserves attention, because regional differences in drinking water hardness could lead to differences in trace element uptake in large parts of the population.

This should be seen in relation to the epidemiologically found negative correlation between mortality from cardiovascular diseases and drinking water hardness, i.e., more people dying of heart disease in soft than in hard water regions. It cannot be excluded that this correlation is caused by differences in trace element uptake, either due to a different supply of trace elements in the drinking water or to an effect of Ca and Mg in hard drinking water on trace element absorption. The latter effect could be based upon a direct interaction between Ca and Mg and trace elements on the level of absorption, but also on an indirect influence of the capacity of the organism for absorption and retention through the Ca- and Mg-status.

In a series of experiments (5.2-5.4) in which the experimental conditions were chosen on ground of the fundamental knowledge obtained in the first part of the study, the influence of Ca and Mg in drinking water and food and that of the Ca- and Mg-status on the uptake of the seven elements mentioned earlier (Zn, Cu, Co, Cd and Se: 5.2; Mn: 5.3; Pb and Cd: 5.4) was tested in mice. For some elements interesting effects were found. The absorption of Pb from drinking water was reduced by Ca, that of Mn and Co from drinking water by both Ca and Mg. In Ca-deprived mice a strongly increased absorption of Mn and Pb and a reduced absorption of Co were found.

As a consequence the uptake of Mn, Co and Pb could be significantly increased in soft water areas compared to hard water areas. With respect to Mn and Co it may be assumed that the body will react to differences in uptake by adaptation of the excretion (homeostasis), so the ultimate retention of these elements will only slightly be influenced. A homeostatic regulation of the retention of Pb is not likely. As toxic manifestations of Pb occur already at low intake, an increased uptake in soft water regions could have consequences for public health.

A high mortality was observed in mice fed a Mg-deficient diet for several weeks (55). This mortality was not found, however, in Mg-deficient mice that were simultaneously deprived of Ca. Through demineralization of the bone Mg-deficiency could lead to Ca accumulation in soft tissues (particularly kidneys and heart). This Ca accumulation could be reduced by simultaneous Ca deprivation. In the literature Mg is mentioned as an important protecting factor against cardiac disease. Mg in hard drinking water could be the "water factor" responsible for the epidemiologically found negative correlation between drinking water hardness and cardiovascular disease. Therefore, in central softening of drinking water removal of Mg should be avoided. Complete removal of Ca from the drinking water could also have a negative effect on public health because of an increased uptake of Pb from the water. Therefore, the Ca concentration in the drinking water should be maintained on a minimal level.

ABSORPTIE EN RETENTIE VAN SPOORELEMENTEN IN DE MUIS
De rol van experimentele condities en de invloed van Ca en Mg.

SAMENVATTING

Spoorelementen zijn de vitamines van de jaren 80. De sterk toegenomen interesse van de medicus, biochemicus en toxicoloog voor de biologische rol van spoorelementen komt tot uiting in het toenemende aantal internationale symposia over spoorelementen, de verschijning van nieuwe wetenschappelijke tijdschriften en duidelijke accentverschuivingen in de bestaande literatuur. Na een eerste golf van voornamelijk kwalitatief onderzoek naar de biologische rol van spoorelementen wordt nu vooral ook aandacht besteed aan kwantitatieve aspecten. Deficienties, zowel klinisch als subklinisch, van als essentieel bekend staande elementen (Zn, Cu, Se, enz.) en belasting met als toxisch bekend staande elementen vanuit het milieu (Cd, Pb, As, enz.) zijn onderwerp van veel onderzoek.

De termen "essentieel" en "toxisch" blijken betrekkelijk te zijn: alle "essentiele" elementen zijn in grote hoeveelheden toxisch en vele "toxische" elementen blijken uiteindelijk ook essentieel te zijn. De betekenis van een spoorelement voor een organisme wordt dus o.a. bepaald door de hoeveelheid, welke in het lichaam terecht komt. Dit is enerzijds afhankelijk van het aanbod en de beschikbaarheid van het element, anderzijds van het vermogen van het organisme om het element op te nemen en vast te houden. Verstoringen van de spoorelementbalans zijn dan ook altijd op deze aspecten terug te voeren.

De bestudering van de absorptie en retentie van radioactief gemerkte spoorelementen in de muis en met name van de condities (o.a. de aanwezigheid van Ca of Mg), welke deze absorptie en retentie kunnen beinvloeden, staat centraal in dit proefschrift. De resultaten van spoorelement studies worden beinvloed door de experimentele condities, waaronder de resultaten verkregen zijn. Het probleem is, dat sommige condities niet onderkend worden als factoren die het eindresultaat van het experiment beinvloeden. Het eerste deel van het onderzoek beoogde dan ook om meer inzicht in dit facet van spoorelementonderzoek te verkrijgen, opdat enerzijds experimenten doelgerichter en met toenemende reproduceerbaarheid opgezet kunnen worden, anderzijds experimenten welke in condities verschillen, beter met elkaar vergeleken kunnen worden.

De basis van spoorelementonderzoek is het gebruik van een geschikt proefdierdieet, dat constant van samenstelling is, een adekwaat maar niet excessief mineraal- en spoorelementgehalte heeft en zo min mogelijk spoorelementen complexerende componenten bevat. Een dergelijk dieet (TRI-CB gezuiverd dieet) werd ontwikkeld en getest (4.2) en gedurende het gehele

onderzoek gebruikt

De invloed van dit dieet op de spoorelementabsorptie bij de muis werd onderzocht voor zeven elementen, te weten Zn, Cu, Mn, Co, Pb, Cd en Se (4 3) Na voeding gedurende twee weken met dit dieet werden voor de meeste elementen veranderingen in de absorptie gevonden. Blijkbaar past de stofwisseling zich aan bij de lagere spoorelementopname uit het gezuiverde dieet Voor één testelement, Zn, werd de meest geschikte aanpassingsperiode voor de muis bepaald (2 weken). Deze periode werd gedurende het gehele onderzoek aangehouden.

Met het spoorelement Zn werden tevens verschillende methoden van spoorelementtoediening getest en werd de invloed van vasten vóór, tijdens en na spoorelementtoediening onderzocht (4.4). Hierbij bleek, dat toediening van een Zn-oplossing met een maagsonde de toediening van Zn in drinkwater kan vervangen, maar dat toediening van Zn in voedsel de absorptie sterk verlaagt. Hetzelfde effect wordt bereikt door consumptie van voedsel vlak voor, tijdens of direct na toediening van een Zn-oplossing. Dit voedseleffect stelt hoge eisen aan de reproduceerbaarheid van voedingscondities tijdens absorptie experimenten.

De absorptie en retentie van Cu, Zn, Se en Pb bleken in het algemeen afhankelijk te zijn van de concentratie, waarin deze elementen werden toegediend (4 5) Ook dit is derhalve een factor, welke weloverwogen gekozen dient te worden. De verkregen resultaten boden tevens een goed inzicht in de verschillende manieren, waarop de muis de spoorelementbalans reguleert (homeostase).

De darmpassage van Cu en Zn werd onderzocht in zowel muis als rat (4 6). Meer inzicht werd hierdoor verkregen in het element-specifieke karakter van de darmpassagesnelheid en de mate van absorptie en in de invloed van voedselconsumptie na spoorelementtoediening op deze factoren.

Tot slot werden de lichaamsverdelingen van intraveneus en intraperitoneaal toegediend Zn met elkaar vergeleken en vrijwel identiek bevonden (4 7)

Met behulp van de verkregen kennis en vaardigheid in het uitvoeren van absorptie en retentie experimenten was het mogelijk het tweede deel van het onderzoek uit te voeren, namelijk bestudering van de invloed van Ca en Mg op de opname van spoorelementen uit voedsel en drinkwater Deze invloed is van belang, omdat door toedoen hiervan regionale verschillen in drinkwaterhardheid kunnen leiden tot verschillen in spoorelementopname in bevolkingsgroepen.

Dit moet gezien worden tegen de achtergrond van een epidemiologisch gevonden negatieve correlatie tussen mortaliteit t.g.v. hart- en vaatziekten en drinkwaterhardheid. Deze correlatie suggereert, dat in gebieden met zacht drinkwater meer mensen aan hart- en vaatziekten overlijden dan in gebieden met hard drinkwater ten gevolge van het verschil in waterhardheid. Het is niet uitgesloten, dat een dergelijke correlatie veroorzaakt wordt door ver-

schillen in spoorelementopname, hetzij door een verschillend aanbod in het drinkwater, hetzij door een effect van Ca en Mg uit hard drinkwater op de absorptie. Het laatstgenoemde effect zou zowel gebaseerd kunnen zijn op een directe interactie tussen Ca en Mg en spoorelementen op absorptieniveau als op een indirecte beinvloeding van het absorptie- en retentievermogen van het organisme via de Ca- en Mg-status.

In een reeks van experimenten (5.2-5.4), waarvan de condities gekozen waren op grond van de in het eerste deel van het onderzoek verkregen fundamentele kennis omtrent dit soort experimenten, werd de invloed van Ca en Mg in drinkwater en voedsel en van de Ca- en Mg-status getest op de opname van de zeven eerder genoemde elementen door de muis (Zn, Cu, Co, Cd en Se: 5.2; Mn: 5.3; Pb en Cd: 5.4). Voor enkele elementen werden interessante effecten gevonden. De absorptie van Pb uit drinkwater werd geremd door Ca, de absorptie van Mn en Co uit drinkwater door zowel Ca als Mg. In muizen met een lage Ca-status werd een sterk toegenomen absorptie van Mn en Pb en een afgenomen absorptie van Co gevonden.

De konsekwentie hiervan is dat in gebieden met zacht water de opname van Co, Mn en Pb aanzienlijk verhoogd kan zijn t.o.v. gebieden met hard water. Ten aanzien van Mn en Co mag verwacht worden, dat het lichaam op verschillen in opname zal reageren met een aangepaste excretie (homeostase), waardoor de uiteindelijke retentie van de elementen niet sterk beinvloed wordt. Een dergelijke regulatie van de retentie van Pb is niet aannemelijk. Aangezien de toxiciteit van Pb zich reeds manifesteert bij zeer lage opname, zou een verhoging van de opname in zacht water gebieden konsekwenties kunnen hebben voor de volksgezondheid.

Onder Mg-deficiente muizen werd een hoge mortaliteit waargenomen (5.2, 5.5). Deze trad niet op onder muizen die niet alleen Mg-, maar ook Ca-deficient waren. Mg-deficientie leidt via botdemineralisatie tot Ca stapeling in nier- en hartweefsel. Deze stapeling wordt afgeremd door gelijktijdige Ca-deficientie. Mg komt uit de literatuur naar voren als een belangrijke beschermende factor tegen hartziekte. De aanwezigheid van Mg in hard drinkwater zou de "water factor" kunnen zijn, welke aan de basis ligt van de epidemiologisch gevonden negatieve correlatie tussen drinkwaterhardheid en hartziekte. Behoud van Mg in drinkwater bij centrale water ontharding lijkt gewenst. Volledige verwijdering van Ca uit drinkwater zou een negatieve invloed op de volksgezondheid kunnen hebben door een verhoging van de opname van Pb uit het drinkwater. Behoud van een zekere hoeveelheid Ca in drinkwater lijkt eveneens gewenst.

NAWOORD

Op deze plaats wil ik dank zeggen aan mijn ouders, die mij in de gelegenheid stelden om te studeren, en aan mijn broer Wim voor zijn voortdurende belangstelling en aanmoediging.
Velen hebben mij in de afgelopen jaren terzijde gestaan bij de uitvoering van mijn werkzaamheden op het IRL. Een aantal van hen heb ik reeds bedankt voor hun bijdrage aan het onderzoek in de bij de artikelen vermelde "Acknowledgements" In het bijzonder wil ik hier mijn dank uitspreken aan de drie stagiairs van het Van Leeuwenhoek Instituut in Delft, welke ieder een jaar aan het onderzoek hebben meegewerkt, namelijk Henrie Terlouw, Erwin Oosterling en Casper Witteman TH studente Margot Wijnen wil ik bedanken voor het ontwikkelingswerk aan de telapparatuur voor kleine proefdieren. De heer Morse leverde een belangrijke bijdrage aan het onderzoek door de verzorging van de gezuiverde proefdiervoedsels. Gert Jan van den Berg dank ik voor zijn zorg voor de proefdieren, met name in de weekeinden en vakanties.
Voor het onderzoek zijn vele proefdieren opgeofferd. Ik hoop dat de resultaten van het onderzoek deze opoffering rechtvaardigen.
Mijn collega's dank ik voor hun begrip voor mijn onregelmatige werktijden en het ongemak dat dit af en toe met zich mee bracht. Omdat kleine dingen soms belangrijk zijn, dank ik Jan van Dop voor zijn dagelijkse vrolijke groet op de gang.
Een bijzonder woord van dank geldt Prof. ir. J.P.W. Houtman voor zijn uitgebreide adviezen ter verbetering van de kwaliteit en leesbaarheid van het proefschrift. De tekeningen in dit proefschrift werden uitstekend verzorgd door Nita Brands. De fotografie was in de deskundige handen van de heer F Hammers van de afdeling der Scheikundige Technologie van de TH Delft. Marlène Beunis en Pieter den Ouden waren zeer behulpzaam bij het corrigeren van de tekst.
Tot slot dank ik Hans Prins en mijn huisgenoten Arco van der Toorn en Paul van Catz voor hun bemoedigende woorden in moeilijke tijden. Verder dank ik allen die ik liefheb, omdat zij de motor van mijn leven zijn.

CURRICULUM VITAE

Overeenkomstig de wens van de Faculteit der Geneeskunde volgt hier een korte levensloop van de schrijver.

Direct na het behalen van het eindexamen Gymnasium-β aan het St.-Bonaventura Lyceum te Leiden in 1970 begon hij zijn studie in de Scheikundige Technologie aan de Technische Hogeschool te Delft. Het kandidaatsexamen (hoofdrichting Biochemie) legde hij af in 1975. In datzelfde jaar verrichtte hij gedurende een half jaar onderzoek op de afd. Radiobiologie van het Studie Centrum voor Kernenergie (SCK) in Mol, België, o.l.v. Prof. O. van der Borght, aan de stofwisseling van ^{85}Sr in muizen.

Na deze stageperiode bereidde hij zich voor op het ingenieursexamen, dat hij in 1977 behaalde. Zijn afstudeeronderzoek verrichtte hij binnen de onderzoeksgroep Nucleaire Biotechniek van het Interuniversitair Reactor Instituut (IRI) in Delft o.l.v. Prof. ir. J.P.W. Houtman en de onderzoeksgroepleider Dr. C.J.A. van den Hamer. Dit onderzoek omvatte de bestudering van zink bindende eiwitten in de lever van de rat en de Zn-Cu interactie in het maagdarmkanaal van de rat tegen de achtergrond van de ziekte van Wilson.

Na het ingenieursexamen was hij van 15 oktober 1977 tot op heden in dienst van het IRI, alwaar hij binnen de onderzoeksgroep Nucleaire Biotechniek onder toezicht van Prof. Houtman en Dr. van den Hamer het onderzoek voor dit proefschrift verrichtte. Dit onderzoek werd vanaf 1 december 1979 tot 1 december 1983 financieel ondersteund door het Ministerie van Volksgezondheid en Milieuhygiene.

De schrijver verhuisde in 1970 vanaf zijn geboorteplaats Leiden naar Delft. Hij is niet getrouwd en heeft waarschijnlijk geen kinderen.

APPENDIX I

DE INVLOED VAN FYTAAT IN DE VOEDING
EEN OVERZICHT

Inleiding

Bij een toenemend gebruik van fytaathoudende produkten in het voedingspakket (ruwe granen, zemelen, soja) verdient de invloed van fytaat op de beschikbaarheid van mineralen en spoorelementen ruime aandacht. Dit geldt met name voor vegetarische diëten, waarin juist deze produkten vaak een belangrijke rol vervullen en waarin vlees, een bron van goed beschikbare spoorelementen, ontbreekt. Een ander kritisch terrein is het gebruik van soja in babyvoeding (bijv. voor kinderen met lactose-intolerantie). Hier is de aanwezigheid van fytaat in de voeding gecombineerd met een laag spoorelement-gehalte en een hoge behoefte aan elementen ten behoeve van groeiprocessen. In dit artikel wordt een overzicht gegeven van de huidige literatuur op het gebied van fytaat en de beïnvloeding van de mineraal- en spoorelement-stofwisseling door fytaathoudende voedingsmiddelen.

Chemie

Fytinezuur is een wijdverbreid in de (planten)natuur voorkomend polyfosforzuur, of beter, een poly-gefosforyleerd inositol. De 6 hydroxylgroepen van inositol zijn verestерd met fosforzuur groepen en de juiste naam is 1,2,3,4,5,6-hexakis (diwaterstof fosfaat) myo-inositol (zie fig. 1). De 6 fosfaatgroepen kunnen bindingen aangaan met 1-, 2- of 3-waardige ionen tot een totaal valentie van 12, waarbij fytinezout ofwel fytaat gevormd wordt.

Het fytaat zoals we dat in de natuur aantreffen is meestal een Ca,Mg-fytaat en wordt wel "fytine" genoemd. In tegenstelling tot Na-fytaat zijn de Ca- en Mg-zouten van fytaat onoplosbaar. Ze vertonen een grote affiniteit voor (2-waardige) metaalionen, zoals Cu, Zn, Mn, Fe, Mo, Co, Cd en Pb. Ook de zouten van 2- en 3-waardige metalen zijn slecht oplosbaar, behalve bij lage pH, waarbij dissociatie optreedt (zie verder hfdstk: Fytaat-metaal interacties in vitro).

Vóórkomen

Fytaat komt voor in een grote varieteit planten, in het bijzonder granen (tarwe, haver, gerst, mais, rijst), peulvruchten (met name sojabonen), noten en zaden (sesamzaad, zonnebloemzaad, koolzaad). De concentratie kan hierbij oplopen tot 5%. Het komt niet voor in de meeste fruitsoorten, bladgroenten, uien en paddestoelen; aardappelen en wortelsoorten kunnen wel lage gehalten

PHYTIC ACID

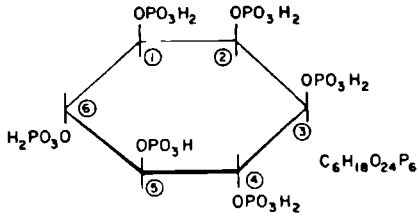


Fig. 1 De chemische structuur van fytinezuur (uit: ref. B).

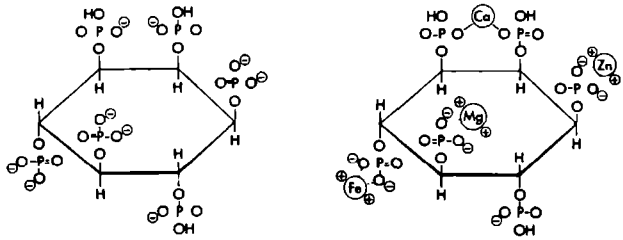


Fig. 2 De chemische binding van mineralen en spoorelementen in fytaat (uit: ref. B).

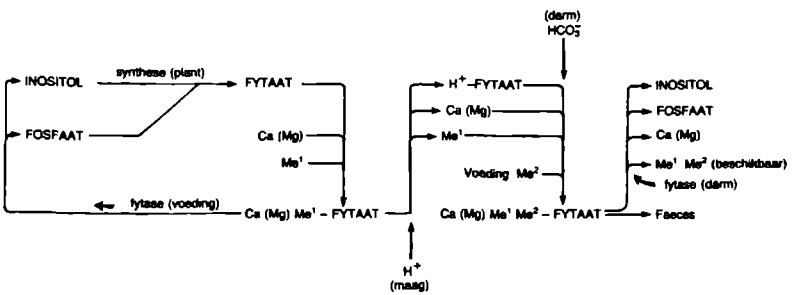


Fig. 3 Het mechanisme van de binding van metalen (Me^1, Me^2) door fytaat, in de plant en in het maagdarmkanaal.

bevatten. De locatie van het fytaat varieert. In sojabonen is het gelijk verdeeld, echter in tarwe bevindt het zich voornamelijk in de zemel en in mais in de kiem. Fytaatgehalten in voedingsprodukten kunnen hoger zijn dan in de uitgangsstof door selectie van specifieke fytaat-bevattende fracties. Enkele gehalten in veel voorkomende produkten zijn weergegeven in Tabel 1.

Het fytaatgehalte van de graankorrel varieert afhankelijk van het rijpingsstadium van de korrel. Het fytaat wordt gevormd tijdens de rijping, waarschijnlijk als een opslagvorm van fosfaat, en wordt tijdens de ontkiemung weer afgebroken, waarbij het opgeslagen fosfaat vrijkomt. Op het toppunt van de fytaatvorming bevindt 80% van de fosfor zich in de vorm van fytaat. Fytaat kan gedeeltelijk complex gebonden zijn aan proteinen.

Analyse

De traditionele bepaling van fytaat berust op extractie uit de grondstof met HCl en precipitatie van Fe(III)-fytaat met $FeCl_3$. Het precipitaat wordt gehydrolyseerd met zwavel- en salpeterzuur, waarna het fosfor- of Fe-gehalte colorimetrisch bepaald wordt. Soms wordt de overmaat van een bekende hoeveelheid toegevoegd $FeCl_3$ colorimetrisch gemeten om hieruit het als fytaat geprecipiteerde Fe en derhalve het fytaatgehalte te berekenen. In een recentere methode wordt de overdracht van Fe van sulfosalicylzuur naar fytaat colorimetrisch gemeten [1]. Een aantal colorimetrische methoden zijn onderling vergeleken, waarbij aan de analyse van geprecipiteerd fytaat-P de voorkeur werd gegeven [2]. Onlangs zijn voor de bepaling van geextraheerd fytaat ook HPLC-methoden ontwikkeld met een aanzienlijk hogere gevoeligheid dan de colorimetrische methoden [3,4]. Een ander voordeel van HPLC is, dat de moeilijk controleerbare precipitatiestap, waarin ook andere polyfosfaten en soms anorganische fosfaten mede geprecipiteerd worden, achterwege blijft.

Tabel 1 Fytaatgehalten in voedingsmiddelen [88].

Produkt	% fytaat
Soja bonen	1.87 - 2.58
Soja babyvoeding	1.00 - 1.14
Tarwe meel	0.68 - 1.23
Tarwe zemelen	4.53
Infant cereals	1.30 - 1.45

Fytaat-metaal interacties in vitro

Fytaat heeft een grote affiniteit voor metaalionen, waarbij de oplosbaarheid afneemt met toenemende waardigheid van de gebonden ionen. Bij pH 7.4 vormt Na-fytaat complexen met (in volgorde van afnemende affiniteit) Cu(II), Zn, Ni, Co, Mn, Fe(III) en Ca [5]. Precipitatie van fytaat met Ca of Mg geeft aanleiding tot coprecipitatie van aanwezige metalen. Vooral Zn, maar ook Cu en Mn worden met een grote efficientie uit een fytinezuur-oplossing neergeslagen, wanneer Ca- of Mg-zouten worden toegevoegd [6]. Het precipitaat bestaat waarschijnlijk uit een netwerk van onderling door Ca en Mg verbonden fytaatmoleculen, waartussen de metaalionen ingevangen zijn. Bij toevoegen van metaalionen aan een Ca-fytaat suspensie vindt binding van de metalen aan het fytaat plaats, door uitwisseling tegen Ca-ionen of door opvulling van nog onbezette bindingsplaatsen. Een affiniteitsreeks ten opzichte van metalen, zoals gevonden voor fytaat in oplossing, is echter niet bekend voor onoplosbaar Ca- of Mg-fytaat.

In de natuur liggen de verhoudingen tussen fytinezuur, mineralen en metalen zodanig, dat fytaten steeds aanwezig zullen zijn als Ca,Mg-zouten (fytine), waarin slechts een fractie van de bindingsplaatsen bezet is door metalen. Pure metaalzouten komen waarschijnlijk niet voor.

Fytaat-metaal interacties in vivo

Een groot aantal experimenten heeft aangetoond, dat toevoeging van Na-fytaat aan de voeding bij de rat de beschikbaarheid van Zn doet afnemen [6-11]. In het darmlumen kan Zn uit het voedsel (exogeen Zn) door fytaat zodanig gebonden worden, dat het niet meer beschikbaar is voor absorptie. Er zijn eveneens aanwijzingen voor binding van door het lichaam in het darmlumen uitgescheiden Zn (endogeen Zn) door fytaat [7]. Naast Zn wordt ook de absorptie van Cu [7], Mn [7], Fe [7], Cd [12] en Pb [12,13] door fytaat geremd. Wanneer de Fe-opname bekeken wordt aan de hand van de haemoglobine-produktie, is geen effekt van fytaat op Fe waarneembaar [14,15]. Fe1-fytaat wordt zelfs wel beschouwd als een goed beschikbare Fe-bron; echter Fe2-fytaat en Fe4-fytaat zijn slecht beschikbaar [16].

De mate waarin de beschikbaarheid van Zn afneemt, wordt niet alleen bepaald door het fytaat- of Zn-gehalte als zodanig, maar ook door het Ca-gehalte in het voedsel en de fytaat/Zn-verhouding [6,9,10]. De drempelwaarde van deze verhouding, waarbij een effekt van fytaat op de Zn-stofwisseling gevonden wordt, bedraagt 12 voor 0.75% Ca in het dieet, resp. 6 voor 1.75% Ca in het dieet [9]. Ca is nodig om een onoplosbaar en dus niet absorbeerbaar complex met Zn te vormen, dat bovendien in zekere mate bestand is tegen enzymatische afbraak door fytase (zie hfdstk: Fytase). De fytaat/Zn-verhouding wordt (in combinatie met het Ca-gehalte) wel als indica-

tor beschouwd voor de Zn-beschikbaarheid in het dieet. Indien het Zn-gehalte in het dieet enkele malen groter is dan de minimale behoefte, zullen ook bij hogere fytaat/Zn-verhoudingen geen Zn-tekorten optreden [9].

Vrijwel alle onderzoek met Zn en gezuiverd fytaat is verricht met ratten. Er is slechts een humane studie bekend [17]; hierin veroorzaakte orale toediening van Zn in combinatie met fytaat een geringere stijging van het serum Zn dan Zn alleen. Toevoeging van Ca als gluconaat gaf een verbetering van de Zn absorptie te zien in plaats van de te verwachten verslechtering. Vervanging van fytaat door bifosfaat gaf geen verschil in de plasma respons. Het is de vraag of het fytaat in dit experiment ook als zodanig werkzaam is geweest of enzymatisch door fytase is afgebroken tot fosfaat.

Het effekt van fytaat op de Ca-stofwisseling (hypocalcemia) is minder goed bekend. Volgens sommigen is Ca-gebrek niet zozeer het gevolg van een verminderde inname van Ca door fytaat als wel van een verhoogde opname van fosfaat, dat vrijkomt bij afbraak van fytaat door fytase [18]. Een andere hypothese luidt, dat fytaat uit de darm opgenomen wordt en vitamine D bindt en zo het Ca-metabolisme verstoort [19].

Er is gesuggereerd, dat fytaat het cholesterolgehalte in bloed verlaagt door de verhouding tussen de Cu- en Zn-absorptie te wijzigen [20]. De relatie tussen cholesterol en de Cu/Zn-verhouding in het dieet is in het verleden gepostuleerd als een oorzakelijke factor in de etiologie van hart- en vaatziekten [21]. Bovengenoemde theorie zou een verklaring geven voor het beschermende effekt van (fytaatrijke) zemelen voor het hart- en vaatstelsel.

Conclusie: Uit de in vivo experimenten met gezuiverd fytaat komt vooral de toxische rol van fytaat als remmer van de Zn-absorptie naar voren, terwijl in tweede instantie ook de remming van andere essentiele metalen genoemd wordt. Daarnaast heeft fytaat ook een beschermende rol als remmer van de absorptie van de toxische metalen Cd en Pb.

Fytaat in de voeding

1. Geschiedenis.

De rol van fytaat als voedselcomponent welke de beschikbaarheid van mineralen en metalen vermindert, is reeds bekend sinds de jaren 40, waarin fytaat herkend werd als de veroorzaker van rachitis-achtige verschijnselen bij dieren. In diezelfde tijd werd een beperkte beschikbaarheid van Fe gevonden bij consumptie van fytaatrijk bruin brood. Fytaat kwam echter pas echt in de belangstelling, nadat de invloed op de opneembaarheid van Zn bekend was geworden.

Rond 1960 kwamen O'Dell en anderen tot de conclusie dat soja in de voeding van pluimvee aanleiding gaf tot Zn-deficientie ten gevolge van het hoge fytaatgehalte van sojabonen [22]. Een beperkte beschikbaarheid van Zn werd

vervolgens aangetoond in een reeks van fytaat bevattende plantaardige voedingsstoffen [23] Het werd ook al snel duidelijk, dat Ca een belangrijke rol speelde bij de Zn-binding door fytaat [24,25].

Het onderzoek van Reinhold naar de oorzaak van Zn-deficiëntie in de plattelandsbevolking van Iran toonde een verband aan met de consumptie van een ongegist type volkorenbrood (tandk) met een hoog zemelen-gehalte [26]. Deze ontdekking heeft aanleiding gegeven tot een grote hoeveelheid onderzoek naar de invloed van zemelen op de beschikbaarheid van mineralen en spoorelementen. De volgende paragrafen zullen met name de fytaat-aspecten van soja en van graanprodukten (brood, zemelen, ontbijtgranen) behandelen.

2. Fytaat in soja.

Sojabonen hebben een hoog fytaatgehalte, gelijkelijk verdeeld over de gehele boon. Afhankelijk van de bereidingsprocedure kunnen sojaprodukten (sojameel, soja-eiwitextract, sojavlees) eveneens veel fytaat bevatten. Aangezien soja veel gebruikt wordt in diervoeding, vaak in combinatie met hoge Ca-gehalten, is in deze sector de fytaat-problematiek met betrekking tot de Zn-stofwisseling het eerst herkend. Men heeft dit probleem voorlopig opgelost door aan soja bevattende diervoeding extra Zn toe te voegen. Er wordt echter nog steeds gezocht naar een goede methode om fytaat uit soja te verwijderen zonder de voedingswaarde van het produkt aan te tasten. Vanwege dit laatste argument wordt bijv. autoclaveren van soja (thermische afbraak van fytaat) ongeschikt geacht

In de laatste jaren heeft soja hernieuwde belangstelling gekregen als eiwitbron in de humane voeding, met name als vervanger van vlees en als onderdeel van babyvoeding. Bestudering van een aantal sojavlees produkten (TVP - textured vegetable protein) [27] toonde aan, dat deze produkten een hoog fytaatgehalte (1-2%) en een hoge fytaat/Zn-verhouding (25-42) hebben, in ratten gevoed met deze produkten werden een verlaagde groeisnelheid en lage plasma Zn waarden gevonden in vergelijking met ratten gevoed met ei-albumine als eiwitbron. De beperkte Zn-beschikbaarheid in soja-eiwit bevattende diëten betreft voornamelijk het intrinsiek in de soja aanwezige Zn, het aan de diëten toegevoegd Zn (extrinsiek Zn) blijft redelijk beschikbaar [28,29]. In Zn-arm gemaakte ratten wordt echter een duidelijke remming van de absorptie van extrinsiek Zn door soja-eiwit (in vergelijking met ei-eiwit) gevonden [10].

Een onderzoek met brood op soja-eiwit basis toonde aan, dat de ingredienten voor het bakken de Zn-beschikbaarheid beperkten, maar dat in het gebakken brood dit effekt niet optrad, waarschijnlijk door fytase activiteit tijdens de gisting en thermische afbraak van fytaat tijdens het bakken [30].

In twee humane studies werd geen effekt van soja als vervanger van vlees op de Zn-balans gevonden [31,32]. De absorptie van Zn uit op soja-eiwit

gebaseerde babyvoeding was zowel in ratten [33,34] als in mensen [35] verlaagd ten opzichte van Zn-absorptie uit op melk gebaseerde babyvoeding. Beiden zijn overigens sterk verlaagd ten opzichte van moedermelk, ook als Zn-bron blijft moedermelk dus te prefereren boven kunstmatige voeding. In een studie met babies op Jamaica werden optredende groeistoornissen met name geweten aan Zn-gebrek ten gevolge van het gebruik van op soja gebaseerde babyvoeding [36].

Dierproeven met Ca en Mg toonden aan, dat de beschikbaarheid van Ca niet en van Mg nauwelijks (alleen bij zeer hoge fytaatgehalten) beperkt is in soja bevattende diëten [29,37]. Er is geen verband tussen fytaat in soja en de beschikbaarheid van Fe voor de rat [38-40]. Integendeel, het hoge Fe-gehalte en de goede beschikbaarheid van dit Fe maakt soja tot een goede Fe-bron voor de rat [39]. In de mens daarentegen wordt de Fe-absorptie sterk geremd door soja-eiwit [41,42], toevoeging van bijv. ascorbinezuur aan soja, met name in babyvoeding, is gewenst om de Fe-beschikbaarheid te verbeteren [43]. Een andere mogelijkheid, toevoeging van extra Fe, heeft als nadeel een mogelijke remming van de Zn-absorptie door Fe/Zn-interactie [44] en is daardoor minder wenselijk.

Conclusie: Een negatief effekt van soja op de Zn-absorptie komt vooral naar voren uit rattenproeven. In de humane voeding blijkt soja als vlees-substituut geen problemen te geven voor de metaal-absorptie, waarschijnlijk vanwege de relatief geringe bijdrage van soja aan het totale dieet. Soja in babyvoeding is echter een slechte bron van Zn en Fe, zodat suppletie van Zn en ascorbinezuur (ter bevordering van de Fe-absorptie) gewenst is.

3 Fytaat in graanprodukten (brood, zemelen en ontbijtgranen)

De beschikbaarheid van Zn in verschillende graansoorten varieert van 40 tot 75% ten opzichte van Zn sulfaat, afhankelijk van het fytaatgehalte en van de fytaat/Zn-verhouding in de granen [45]. Verbetering van de beschikbaarheid kan bereikt worden door extra Zn aan het graanprodukt toe te voegen, alsook door de voedingsbodem van het graan met Zn te bemesten, waardoor het intrinsieke Zn-gehalte van het graan toeneemt [46].

Het belangrijkste graanprodukt is brood. De laatste jaren hebben in Nederland een aanzienlijke verandering in de broodconsumptie te zien gegeven. De belangstelling is verschoven van het smaakarme fabriekswitbrood naar het volkorenbrood van de "warme bakker". Hiermee gaat gepaard een toegenomen consumptie van zemelen, de fytaat bevattende fractie van de tarwekorrel. Zelfs worden zemelen als aparte fractie aan de voeding toegevoegd ter verhoging van het vezelgehalte van de voeding (dit zou de stoelgang bevorderen, een heilzame werking hebben op hart en bloedvaten en tevens de kans op darmkanker verlagen). Tot op heden is zeer weinig aandacht besteed aan de konsekwenties

van deze zemelen-suppletie voor de mineraal- en spoorelement-balans. Toch is de meeste informatie over de invloed van fytaat en vezelstof op de humane stofwisseling afkomstig uit onderzoek aan zemelen (met name in volkorenbrood).

Zeer belangrijk en baanbrekend werk is verricht door de groep van Reinhold in Iran, welke het verband onderzocht tussen deficientie verschijnselen van Zn en Ca in de plattelandsbevolking en de consumptie van ongegist volkorenbrood (tanok). Aanvankelijk kwamen zij tot de conclusie, dat het hoge fytaatgehalte van tanok een sterk negatieve invloed op de absorptie van Zn en Ca had [26]. Dit fytaatgehalte is niet alleen hoog vanwege het volkoren karakter van tanok (d.w.z. een hoog zemelen-gehalte), maar ook vanwege het feit dat door het ontbreken van een gistingsprocedure afbraak van fytaat door fytase uit gist, zoals in gegist brood voorkomt, achterwege blijft [47].

In een later stadium van het onderzoek werd eveneens een aanzienlijke rol toegekend aan de metaalbindende capaciteit van vezelstoffen (fiber), welke ook met een hoog gehalte in tanok voorkomen (eveneens in de zemelfractie) [48,49]. Dit heeft geleid tot een (onbeslíste) discussie over de relatieve bijdrage van fytaat en fiber aan de verminderde beschikbaarheid van Ca, Mg en Zn in brood [50-57]. Het is echter wel duidelijk geworden, dat ook fiber een negatieve invloed heeft op metaal-beschikbaarheid door binding van de metalen, met name in de oplosbare fractie, en mogelijk ook door zuiver fysische invloeden op absorptie van nutrienten (inkapseling, versnelde darmpassage, etc.). Het wordt echter betwijfeld of deze effekten sterk genoeg zijn om de metaalbalans te verstoren.

Het werk van Reinhold heeft aanzet gegeven tot een grote hoeveelheid onderzoek naar de invloed van volkorenbrood, resp. zemelen (bran), op de stofwisseling. In rattestudies wordt geen effekt gevonden van zemelen op de beschikbaarheid van Ca [58], maar de beschikbaarheid van Mg, Zn en P neemt af [59]. Dit effekt wordt echter gekompenseerd door het hogere gehalte van deze elementen in de zemelen [60]. Vermindering van fytaat in zemelen tot een fytaat/Zn-verhouding=8 met behulp van fytase doet het Zn-remmend effekt van zemelen teniet [61]. In geen geval werd remming van de Fe-beschikbaarheid door fytaat of fiber in zemelen waargenomen [62-64].

In bavianen werd bij consumptie van een zemelen-rijk dieet een verstoring van de Ca-, Cu- en Zn-balans gevonden [65]. In een groep van 13 mannen en 4 vrouwen was geen effekt van zemelen op het Ca-gehalte in het serum aantoonbaar [66]. In 2 mannen ging de Fe-balans achteruit bij consumptie van bazari, een vezelrijk gegist brood; echter niet bij tanok, het ongegiste broodtype, waarschijnlijk vanwege het hoge Fe-gehalte van dit brood [67]. In een In een groep van 60 mannen en vrouwen werd bij consumptie van een zemelen-rijke maaltijd een afname van de non-haem Fe-absorptie met 50-75% gevonden [68]. Verwijdering van fytaat uit de zemelen met behulp van fytase gaf geen verbetering van de Fe-absorptie. De Fe-remmende faktor in zemelen

moet dus een andere zijn dan fytaat; deze faktor schijnt zich vooral in de oplosbare fractie van de zemel te bevinden.

In een studie met volkorenbrood [69] werd gevonden, dat de absolute absorptie van Zn uit dit brood hoger is dan uit witbrood, dankzij het hogere Zn-gehalte van volkorenbrood; alleen bij gelijkgemaakte Zn-gehalten was de absorptie uit volkorenbrood lager. Toevoeging van Ca in de vorm van melk verbeterde de Zn-absorptie. Hier heeft waarschijnlijk de absorptie bevorderende werking van melkeiwitten een rol gespeeld. In twee andere studies met zemelen [70,71] werden slechts marginale effekten van fytaat gevonden op de balans van Fe, Zn, Mn en Cu, resp. Ca, Mg, Fe, Zn en Cu. Een mogelijk verminderde absorptie van deze elementen door toedoen van zemelen werd steeds gekompenseerd door het verhoogde element-gehalte van de zemel zelf.

Een aparte klasse van graanprodukten vormen de ontbijtgranen ("breakfast cereals"). In "infant cereals" werd geen verband gevonden tussen Zn-beschikbaarheid en fytaat-gehalte, waarschijnlijk omdat dit gehalte te laag (0.1%) is om een effekt te veroorzaken [72]. Vanwege het lage Zn-gehalte van infant cereals wordt wel extra Zn toegevoegd, hetgeen een gunstig effekt heeft op de Zn-status [73]. In breakfast cereals gebaseerd op volle graankorrels ("muesli") zal het fytaat- en fiber-gehalte aanzienlijk hoger zijn dan in infant cereals. In één studie (met ratten) bleek de Zn-beschikbaarheid van een dergelijk ontbijt beter te zijn dan die van infant cereals [74]. Cereals worden meestal geconsumeerd met melkprodukten, waarvan de eiwitten de Zn-absorptie bevorderen [69]. Een negatief effekt van cereals zal hierdoor (deels) gekompenseerd worden. De beschikbaarheid van aan ontbijtgranen toegevoegd Fe hangt af van de chemische vorm van dit Fe. De voor Fe-suppletie meestal gebruikte fosfaten worden slechts matig geabsorbeerd [75].

Er is ook enig onderzoek aan typisch vegetarische diëten gedaan. Deze diëten bevatten over het algemeen veel ruwe granen, zemelen en ook sojabonen en sojavlees. Voor een lacto-ovo-vegetarisch dieet en voor een sojavlees bevattend dieet werden fytaat/Zn-verhoudingen van 4.5 en 7.6 gevonden [76]. Er zijn aanwijzingen voor een enigszins verlaagde Zn-status in vegetariërs [77,78] Het lichaam schijnt zich echter geleidelijk aan te passen aan de verminderde beschikbaarheid van Zn en Fe door een efficienter gebruik van de geabsorbeerde metalen [79,80].

Conclusie: Hoewel de literatuurgegevens niet altijd even goed met elkaar in overeenstemming gebracht kunnen worden, geven zij toch geen reden tot ernstige bezorgdheid over het effekt van zemelen, als zodanig of in volkorenbrood, op de metaalstofwisseling. Hieraan ten grondslag ligt het relatief hoge metaal-gehalte van de zemel zelf, hetgeen kompenseert voor een verminderde beschikbaarheid. Slechts bij gebruik van extreme hoeveelheden zemelen in een onevenwichtig dieet, zoals bij dorpsbewoners in Iran, kunnen negatieve balansen van Ca, Zn en Fe optreden.

Fytase

Een complicerende factor in de beschouwing van de invloed van fytaat in de voeding op de metaal beschikbaarheid is de aanwezigheid - in voedingsprodukten (gist in brood) en in de darm - van fytase, een enzym dat fytaat afbreekt door de fosfaatgroepen van de inositol af te splitsen Hierdoor is de werkzaamheid van fytaat variabel en afhankelijk van de fytase activiteit in de voeding en in het organisme Er zijn aanwijzingen, dat de fytase activiteit in het organisme afhankelijk is van de species (rat vs kip, kalf en mens [81]; rat vs hamster [82]), van de leeftijd [83] (afnemende activiteit bij toenemende leeftijd), van de P-status [84] (hogere activiteit bij P-deficientie) en mogelijk ook van de Zn-status Fytase wordt namelijk wel beschouwd als een speciale vorm van alkalische fosfatase [85], een enzym waarvan de activiteit afneemt bij Zn-deficientie [86,87]. Indien dit voor fytase ook geldt, zou dit kunnen verklaren, waarom een effect van soja-fytaat op de absorptie van extrinsiek Zn alleen waargenomen wordt in Zn-arm gemaakte ratten (zie hfdstk: Fytaat in soja)

De fytase activiteit is eveneens afhankelijk van de chemische vorm van het fytaat. Het natuurlijke eiwit-gebonden fytaat is waarschijnlijk beter bestand tegen enzymatische afbraak dan het gezuiverde fytaat [88] Ca in het dieet beschermt fytaat eveneens tegen afbraak door vorming van een conglomeraat van Ca-fytaat, dat precipiteert en moeilijk toegankelijk is voor het enzyme. De fytaat-hydrolyse in brooddeeg door fytase in gist wordt aanzienlijk onderdrukt door toevoeging van Ca (bijv. als melk) aan het deeg [89]. Daarnaast wordt ook de fytaat-hydrolyse in de darm door Ca onderdrukt, in een experiment met C14-gelabeld fytaat was de C14-absorptie 46% in een Ca-rijk dieet tegen 94% in een Ca-arm dieet [90] Het verschil in fytase activiteit verklaart mogelijk een groot deel van de discrepanties, welke gevonden worden tussen de verschillende onderzoeken met fytaat en fytaathoudende voedingsstoffen.

Een laatste strijdpunt is de lokalisatie van fytase in de darm. Experimenten met steriele ratten hebben uitgewezen, dat in afwezigheid van darmflora geen fytase activiteit aantoonbaar is, hetgeen een aanwijzing is dat de fytase niet uit de rat zelf (darmmucosa, pancreas), maar uit darmbacterien afkomstig is [91]. Men kan zich afvragen of er wat dit aspect betreft eveneens species verschillen zijn.

Conclusie. Fytase is een faktor welke in belangrijke mate de werkzaamheid van fytaat bepaalt en als zodanig tot nu toe enigszins is onderschat

Conclusies

Uit het voorafgaande kunnen een aantal conclusies getrokken worden:

1. De resultaten van dierproeven en die van humane studies met fytaat en fytaathoudende voedingsmiddelen zijn slecht met elkaar vergelijkbaar.
2. Er is een duidelijke discrepantie tussen het gedrag van gezuiverd fytaat, toegevoegd aan het dieet, en natuurlijk fytaat, voorkomende in voedingsmiddelen als soja en zemelen.
3. De gevonden tegenstrijdigheden kunnen verklaard worden vanuit verschillen in de chemische vorm van fytaat en vanuit verschillen in de mate van enzymatische afbraak van fytaat door fytase.
4. Humane studies met fytaathoudende voedingsmiddelen wijzen uit, dat in het gemiddelde voedingspakket geen problemen te verwachten zijn voor de mineraal- en spoorelement-stofwisseling.
5. In extreme diëten, zoals strikt vegetarische diëten gecombineerd met een hoog gebruik van zemelen en sojaprodukten (sojabonen, sojavlees) is een negatieve Fe- en Zn-balans niet onmogelijk. Hiervoor zijn niet alleen fytaat, maar ook fiber en mogelijk nog andere componenten verantwoordelijk. Vaak zijn echter dergelijke diëten rijk aan spoorelementen, hetgeen een goede kompensatie vormt voor een beperkte beschikbaarheid van deze elementen door fytaat of fiber. Er zijn bovendien aanwijzingen, dat in vegetariërs de Fe- en Zn-balans zich aanpast aan verminderde absorptie door middel van een efficiënter gebruik van Zn en Fe.
6. Soja bevattende baby- en kleutervoedingen kunnen de Fe- en Zn-balans verstoren. Toevoeging van Zn en ascorbinezuur ter bevordering van de Fe-absorptie wordt aangeraden. Toevoeging van Fe kan een negatieve invloed op de Zn-absorptie hebben. Hiermee dient ook in andere baby- en kleutervoedingen rekening gehouden te worden.

Referenties

Hieronder volgen enkele belangrijke overzichten, waarin fytaat of de werking van fytaat beschreven zijn. Voor specifieke referenties betreffende in het overzicht genoemde aspecten van fytaat raadplege men de genummerde referentielijst.

A. Maga,J.A.: Phytate: Its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. J. Agric. Food Chem. 30 (1982), 1.

B. Erdman,Jr.,J.W.: Oilseed phytates: Nutritional Implications. J. Am. Oil Chem. Soc. 56 (1979), 736.

C. Cosgrove,D.J.: Inositol phosphates. Their chemistry, biochemistry and physiology. Elsevier, Amsterdam-New York (1980).

D. Solomons,N.W.: Biological availability of zinc in humans. J. Am. Clin. Nutr. 35 (1982), 1048.

E. Anonymous: Phytate and zinc bioavallability. Nutr. Rev. 37 (1979), 365.

F. Davies,N.T.: Anti-nutrient factors affecting mineral utilization. Proc. Nutr. Soc. 38 (1979), 121.

G. Oberleas,D. en Harland,B.F.: Nutritional agents which affect metabolic zinc status. In: Zinc metabolism: current aspects in health and disease. Prasad,A.S. en Brewer,G.J., eds. New York: Alan R. Liss (1977), 11.

H. Oberleas,D. en Prasad,A.S.: Factors affecting zinc homeostasis. In: Trace elements in human health and disease. Vol I. Zinc and copper. Prasad,A.S., ed. New York: Academic Press (1975), 155.

I. Reinhold,J.G., Faradji,B., Abadi,P. en Ismail-Beigi,F.: Binding of zinc to fiber and other solids of wholemeal bread. In: Trace elements in human health and disease. Vol I. Zinc and copper. Prasad,A.S., ed. New York: Academic Press (1975), 163.

1. Latta, M. en Eskin, M.: A simple and rapid colorimetric method for phytate determination. J. Agric. Food Chem. 28 (1980), 1313.
2. Thompson, D.B. en Erdman, Jr., J.W.: Phytic acid determination in soybeans. J. Food Sci. 47 (1982), 513.
3. Camire, A.L. en Clydesdale, F.M.: Analysis of phytic acid in foods by HPLC. J. Food Sci. 47 (1982), 575.
4. Graf, E. en Dintzis, F.R.: High-performance liquid chromatografic method for the determination of phytate. Anal. Biochem. 119 (1982), 413.
5. Vohra, P., Gray, G.A. en Kratzer, F.H.: Phytic acid-metal complexes. Proc. Soc. Exp. Biol. Med. 120 (1965), 447.
6. Davies, N.T. en Olpin, S.E.: Studies on the phytate:zinc molar contents in diets as a determinant of Zn availability to young rats. Br. J. Nutr. 41 (1979), 591.
7. Davies, N.T. en Nightingale, R.: The effects of phytate on intestinal absorption and excretion of zinc, and whole-body retention of Zn, copper, iron and manganese in rats. Br. J. Nutr. 34 (1975), 243.
8. Hathcock, J. en Abraham, C.: Effects of protein, amino acids, phytate and cadmium on zinc absorption in rats. Int. Symp. Trace Elements in Man and Animals (TEMA-3) (1977), 126.
9. Morris, E.R. en Ellis, R.: Effect of dietary phytate/zinc molar ratio on growth and bone zinc response of rats fed semipurified diets. J. Nutr. 110 (1980), 1037.
10. Lo, G.S., Settle, S.L., Steinke, F.H. en Hopkins, D.T.: Effect of phytate:zinc molar ratio and isolated soybean protein on zinc bioavailability. J. Nutr. 111 (1981), 2223.
11. House, W.A., Welch, R.M. en Van Campen, D.R.: Effect of phytic acid on the absorption, distribution, and endogenous excretion of zinc in rats. J. Nutr. 112 (1982), 941.
12. Wise, A. en Gilburt, D.J.: Binding of cadmium and lead to the calcium-phytate complex in vitro. Toxicol. Lett. 9 (1981), 45.
13. Wise, A.: Protective action of calcium phytate against acute lead toxicity in mice. Bull. Environm. Contam. Toxicol. 27 (1981), 630.
14. Cowan, J.W., Esfahani, M., Saljé, J.P. en Azzam, S.A.: Effect of phytate on iron absorption in the rat. J. Nutr. 90 (1966), 423.
15. Hunter, J.E.: Iron availability and absorption in rats fed sodium phytate. J. Nutr. 111 (1981), 841.
16. Ellis, R. en Morris, E.R.: Effect of sodium phytate on stability of monoferric phytate complex and the bioavailability of the iron to rats. Nutr. Rep. Int. 20 (1979), 739.
17. Pécoud, A., Donzel, P. en Schelling, J.L.: Effect of foodstuffs on the absorption of zinc sulfate. Clin. Pharmacol. Ther. 17 (1975), 469.
18. Sasson, A., Etzion, Z., Shany, S., Berlyne, G.M. en Yagil, R.: Growth and bone mineralization as affected by dietary calcium, phytic acid and vitamin D. Comp. Biochem. Physiol. 72A (1982), 43.
19. Van Den Berg, C., Hill, L.F. en Stanbury, S.W.: Inositol phosphates and phytic acid as inhibitors of biological calcification in the rat. Clin. Sci. 43 (1972), 377.
20. Kleway, L.M.: Hypocholesterolemia due to sodium phytate. Nutr. Rep. Int. 15 (1977), 587.
21. Kleway, L.M.: Coronary heart disease: The zinc/copper hypothesis. Am. J. Clin. Nutr. 28 (1975), 764.
22. O'Dell, B.L. en Savage, J.E.: Effect of phytic acid on zinc availability. Proc. Soc. exp. Biol. Med. 103 (1960), 304.
23. O'Dell, B.L., Burpo, C.E. en Savage, J.E.: Evaluation of zinc availability in foodstuffs of plant and animal origin. J. Nutr. 102 (1972), 653.
24. Likuski, H.J.A. en Forbes, R.M.: Mineral utilization in the rat. IV. Effects of calcium and phytic acid on the utilization of dietary zinc. J. Nutr. 85 (1965), 230.
25. Oberleas, D., Muhrer, M.E. en O'Dell, B.L.: Dietary metal-complexing agents and zinc availability in the rat. J. Nutr. 90 (1966), 56.
26. Reinhold, J.G., Nasr, K., Lahimgarzadeh, A. en Hedayati, H.: Effects of purified phytate and phytate-rich bread upon metabolism of zinc, calcium, phosphorus, and nitrogen in man. Lancet I (1973), 283.
27. Davies, N.T. en Reid, H.: An evaluation of the phytate, zinc, copper, iron and manganese contents of, and Zn availability from, soya-based textured-vegetable-protein meat-substitutes and meat-extenders. Br. J. Nutr. 41 (1979), 579.
28. Hardie-Muncy, D.A. en Rasmussen, A.I.: Interrelationships between zinc and protein level and source in weanling rats. J. Nutr. 109 (1979), 321.
29. Forbes, R.M., Weingartner, K.E., Parker, H.M., Ball, R.R. en Erdman, Jr., J.W.: Bioavailability to rats of zinc, magnesium and calcium in casein-, egg- and soy protein-containing diets. J. Nutr. 109 (1979), 1652.
30. Ranhotra, G.S., Lee, C. en Gelroth, J.A.: Bioavailability of zinc in soy-fortified wheat bread. Nutr. Rep. Int. 18 (1978), 487.
31. Gregor, J.L., Abernathy, R.P. en Bennett, O.A.: Zinc and nitrogen balance in adolescent females fed varying levels of zinc and soy protein. Am. J. Clin. Nutr. 31 (1978), 112.
32. Sandström, B. en Cederblad, Å.: Zinc absorption from composite meals. II. Influence of the main protein source. Am. J. Clin. Nutr. 33 (1980), 1778.

33 Momcilovic, B., Belonje, B., Giroux, A. en Shah, B.G.: Bioavailability of zinc in milk and soy protein-based infant formulas. J. Nutr. 106 (1976), 913.

34. Johnson, P.E. en Evans, G.W.: Relative zinc availability in human breast milk, infant formulas, and cow's milk. Am. J. Clin. Nutr. 31 (1978), 416.

35. Casey, C.E., Walravens, P.A. en Hambidge, K.M.: Availability of zinc: Loading tests with human milk, cow's milk, and infant formulas. Pediatrics 68 (1981), 394

36 Golden, B.E. en Golden, M.H.N.: Plasma zinc, rate of weight gain and energy cost of tissue deposition in children recovering from severe malnutrition on a cow's milk or soya protein based diet. Am. J. Clin. Nutr. 34 (1981), 892

37. Lo, G.S., Steinke, F.H. en Hopkins, D.T.: Effect of isolated soybean protein on magnesium bioavailability. J. Nutr. 110 (1980), 829

38. Welch, R.M. en Van Campen, D.R.: Iron availability to rats from soybeans. J. Nutr. 105 (1975), 253.

39. Steinke, F.H. en Hopkins, D.T.: Biological availability to the rat of intrinsic and extrinsic iron with soybean protein isolates. J. Nutr. 108 (1978), 481.

40. Rotruck, J.T. en Luhrsen, K.R.: A comparative study in rats of iron bioavailability from cooked beef and soybean protein. J. Agric. Food Chem. 27 (1979), 27

41. Cook, J.D., Morck, T.A. en Lynch, S.R.: The inhibitory effect of soy products on nonheme iron absorption in man. Am. J. Clin. Nutr. 34 (1981), 2622.

42. Morck, T.A., Lynch, S.R., Skikne, B.S. en Cook, J.D.: Iron availability from infant food supplements. Am. J. Clin. Nutr. 34 (1981), 2630

43. Morck, T.A., Lynch, S.R. en Cook, J.D.: Reduction of the soy-induced inhibition of nonheme iron absorption. Am. J. Clin. Nutr. 36 (1982), 219.

44. Anonymous: Inhibition of zinc absorption by inorganic iron. Nutr. Rev. 40 (1982), 76.

45. Lantzsch, H.-J., Schenkel, H. en Nickerl, I.: Zn availability in grains and legumes. Int. Symp. Trace Elements in Man and Animals (TEMA-3) (1977), 460

46. Lantzsch, H.-J., Scheuermann, S.E. en Marschner, H.: Improvement of zinc bioavailability in cereal grains. Int. Symp. Trace Elements in Man and Animals (TEMA-4) (1981), 107.

47. Reinhold, J.G., Parsa, A., Karimian, N., Hamsuck, J.W. en Ismail-Beigi, F.: Availability of zinc in leavened and unleavened wholemeal wheaten breads as measured by solubility and uptake by rat intestine in vitro. J. Nutr. 104 (1974), 976.

48. Reinhold, J.G., Ismail-Beigi, F. en Faradji, B.: Fibre vs. phytate as determinant of the availability of calcium, zinc and iron of breadstuffs. Nutr. Rep. Int. 12 (1975), 75.

49. Reinhold, J.G., Faradji, B., Abadi, P. en Ismail-Beigi, F.: Decreased absorption of calcium, magnesium, zinc and phosphorus by humans due to increased fiber and phosphorus consumption as wheat bread. J. Nutr. 106 (1976), 493.

50. Davies, N.T., Hristic, H. en Flatt, A.A.: Phytate rather than fibre in bran as the major determinant of zinc availability to rats. Nutr. Rep. Int. 15 (1977), 207.

51. Ismail-Beigi, F., Reinhold, J.G., Faraji, B. en Abadi, P.: Effects of cellulose added to diets of low and high fiber content upon the metabolism of calcium, magnesium, zinc and phosphorus by man. J. Nutr. 107 (1977), 510.

52. Ismail-Beigi, F., Faraji, B. en Reinhold, J.G.: Binding of zinc and iron to wheat bread, wheat bran, and their components. Am. J. Clin. Nutr. 30 (1977), 1721.

53. Reinhold, J.G., Garcia L., J.S. en Garzon, P.: Binding of iron by fiber of wheat and maize. Am. J. Clin. Nutr. 34 (1981), 1384.

54. Tsai, R.C.Y. en Lei, K.Y.: Dietary cellulose, zinc and copper: Effects on tissue levels of trace minerals in the rat. J. Nutr. 109 (1979), 1117.

55. Franz, K.B., Kennedy, B.M. en Fellers, D.A.: Relative bioavailability of zinc from selected cereals and legumes using rat growth. J. Nutr. 110 (1980), 2272.

56. McKenzie, J.M. en Davies, N.T.: Dietary protein influences bread effect on zinc in rats. Int. Symp. Trace Elements in Man and Animals (TEMA-4) (1901), 111.

57. Frohlich, W. en Asp, N.-G.: Mineral bioavailability and cereal fiber. Am. J. Clin. Nutr. 33 (1980), 2397.

58. Ranhotra, G.S., Gelroth, J.A., Torrence, F.A., Bock, M.A. en Winterringer, G.L.: Bread (white and whole wheat) and nonfat dry milk as sources of bioavailable calcium for rats. J. Nutr. 111 (1981), 2081.

59. Bagheri, S.M. en Gueguen, L.: Bioavailability to rats of calcium, magnesium, phosphorus and zinc in wheat bran diets containing equal amounts of these minerals. Nutr. Rep. Int. 25 (1982), 583.

60. Bagheri, S.M. en Gueguen, L.: Influence of wheat bran diets containing unequal amounts of calcium, magnesium, phosphorus and zinc upon the absorption of these minerals in rats. Nutr. Rep. Int. 24 (1981), 47.

61. Morris, E.R. en Ellis, R.: Bioavailability to rats of iron and zinc in wheat bran: Response to low-phytate bran and effect of the phytate/zinc molar ratio. J. Nutr. 110 (1980), 2000.

62. Liebman, M. en Driskell, J.: Dietary phytate and liver iron repletion in iron-depleted rats. Nutr. Rep. Int. 19 (1979), 281.

63. Ranhotra, G.S., Lee, C. en Gelroth, J.A.: Bioavailability of iron in some commercial variety breads. Nutr. Rep. Int. 19 (1979), 851.
64. Fairweather-Tait, S.J.: The effect of different levels of wheat bran on iron absorption in rats from bread containing similar amounts of phytate. Br. J. Nutr. 47 (1982), 243.
65. Reman, M.J. en Van Ransburg, S.J.: The influence of a high-bran diet on trace element retention in primates. Phys. Med. Biol. 25 (1980), 433.
66. Harry, R.W., Stout, R.W. en Love, A.H.G.: Lack of effect of bran enriched bread on plasma lipids, calcium, glucose and body weight. Irish J. Med. Sci. 147 (1978), 249.
67. Faraji, B., Reinhold, J.G. en Abadi, P.: Human studies of iron absorption from fiber-rich Iranian flat breads. Nutr. Rep. Int. 23 (1981), 267.
68. Simpson, K.M., Morris, E.R. en Cook, J.D.: The inhibitory effect of bran on iron absorption in man. Am. J. Clin. Nutr. 34 (1981), 1469.
69. Sandström, B., Arvidsson, B., Cederblad, Å. en Björn-Rasmussen, E.: Zinc absorption from composite meals. I. The significance of wheat extraction rate, zinc, calcium, and protein content in meals based on bread. Am. J. Clin. Nutr. 33 (1980), 739.
70. Morris, E.R., Ellis, R. en Steele, P.: Trace element nutriture response of adult men consuming dephytinized or non-dephytinized wheat bran. Trace Subst. Environ. Health 14 (1980), 103.
71. Van Dokkum, W., Wesstra, A. en Schippers, F.A.: Physiological effects of fibre-rich types of bread. 1. The effect of dietary fibre from bread on the mineral balance of young men. Br. J. Nutr. 47 (1982), 451.
72. Shah, B.G., Giroux, A. en Belonje, B.: Bioavailability of zinc in infant cereals. Nutr. Metab. 23 (1979), 286.
73. Hambidge, K.M., Chavez, M.N., Brown, R.M. en Walravens, P.A.: Zinc nutritional status of young middle-income children and effects of consuming zinc-fortified breakfast cereals. Am. J. Clin. Nutr. 32 (1979), 2532.
74. Momcilovic, B. en Shah, B.G.: Bioavailability of zinc in infant foods. Nutr. Rep. Int. 14 (1976), 717.
75. Shah, B.G., Giroux, A. en Belonje, B.: Bioavailability of iron from iron phosphates in cereals and infant foods. J. Agric. Food Chem. 27 (1979), 845.
76. Ellis, R., Morris, E.R., Hall, A.D. en Smith, J.C.: Phytate:zinc molar ratio of regular, ovo-lacto-vegetarian and soy meat substitute hospital diets. Fed. Proc. 40 (1981), 938 (no. 4018).
77. Freeland-Graves, J.H., Greninger, S.A., Young, R.K., Roby, M.J., Friedman, B.J. en Vann, K.L.: Taste acuity in vegetarians and nonvegetarians. Fed. Proc. 40 (1981), 840 (no. 3466).
78. Freeland-Graves, J.H., Lavore Ebangit, M. en Hendrickson, P.J.: Alterations in zinc absorption and salivary sediment zinc after a lacto-ovo-vegetarian diet. Am. J. Clin. Nutr. 33 (1980), 1757.
79. Kies, C., Young, E., McEndree, L., Lo, B.F., Beshgetoor, D. en Fox, H.M.: Zinc utilization by omnivores and vegetarians. Fed. Proc. 40 (1981), 855 (no. 3549).
80. Anderson, B.M., Gibson, R.S. en Sabry, J.H.: The iron and zinc status of long-term vegetarian women. Am. J. Clin. Nutr. 34 (1981), 1042.
81. Bitar, K. en Reinhold, J.G.: Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf, and man. Biochim. Biophys. Acta 268 (1972), 442.
82. Taylor, T.G. en Coleman, J.W.: A comparative study of the absorption of calcium and the availability of phytate-phosphorus in the golden hamster (Mesocricetus auratus) and the laboratory rat. Br. J. Nutr. 42 (1979), 113.
83. Nelson, T.S. en Kirby, L.K.: Effect of age and diet composition on the hydrolysis of phytate phosphorus by rats. Nutr. Rep. Int. 20 (1979), 729.
84. Moore, R.J. en Veum, T.L.: Effect of dietary phosphorus and yeast culture level on the utilization of phytate phosphorus by the rat. Nutr. Rep. Int. 25 (1982), 221.
85. Davies, N.T. en Flatt, A.A.: The similarity between alkaline phosphatase (EC 3.1.3.1) and phytase (EC 3.1.3.8) activities in rat intestine and their importance in phytate-induced zinc deficiency. Br. J. Nutr. 39 (1978), 307.
86. Luecke, R.W., Olsen, M.E. en Baltzer, B.V.: Zinc deficiency in the rat: Effect on serum and intestinal alkaline phosphatase activities. J. Nutr. 94 (1968), 344.
87. Adeniyi, F.A. en Heaton, F.W.: The effect of zinc deficiency on alkaline phosphatase (EC 3.1.3.1) and its isoenzymes. Br. J. Nutr. 43 (1980), 561.
88. Oberleas, D., Muhrer, M.E. en O'Dell, B.L.: The availability of zinc from foodstuffs. In: Zinc metabolism. Ed.: Prasad, A.S., Thomas, Springfield (1966), 225.
89. Zemel, M.B. en Shalef, L.A.: Phytic acid hydrolysis and soluble zinc and iron in whole wheat bread as affected by calcium containing additives. J. Food Sci. 47 (1982), 535.
90. Nahapetian, A. en Young, V.R.: Metabolism of 14C-phytate in rats: Effect of low and high dietary calcium intakes. J. Nutr. 110 (1980), 1458.
91. Wise, A. en Gilburt, D.J.: Phytate hydrolysis by gnotobiotic and conventional rats. Appl. Environ. Microbiol. 43 (1982), 753.

APPENDIX II

ISOTOPE DILUTION IN THE MEASUREMENT OF ^{65}ZN ABSORPTION IN MICE

(This short discussion elaborates part of the discussion in Section 4.5)

Recently, Flanagan et al. [1] reported in mice fed a zinc-deficient diet (1.0 µg Zn/g) a dose dependence for the absorption of ^{65}Zn from food. The ^{65}Zn absorption decreased from about 45% to about 15%, when the Zn dose increased from 6.5 to 130 µg Zn. In mice fed a zinc-replete diet (48.5 µg Zn/g) the ^{65}Zn absorption from food was relatively constant, viz., about 10%. The disappearance of the dose dependence in the latter mice was explained by radioisotope dilution by endogenous luminal zinc.

Isotope dilution effects on ^{65}Zn absorption have been described earlier by Evans et al. [2]. They suggested that ^{65}Zn absorption is not always a good indicator for total zinc absorption, because in case of ^{65}Zn dilution by endogenous zinc the absorption of total zinc is higher than the ^{65}Zn data suggest. It should be realized, however, that ^{65}Zn dilution in the intestine by endogenous luminal zinc will not necessarily lead to a change in ^{65}Zn absorption percentage. This change will only occur when this percentage is dose dependent and the amount of diluting zinc - in comparison to ^{65}Zn - is sufficiently large. If the ^{65}Zn absorption percentage is independent of the dose, then ^{65}Zn dilution by endogenous zinc will not influence ^{65}Zn absorption, though the absorption of total zinc will be increased in proportion to the degree of dilution.

Our own results [3] of Zn absorption studies in mice indicate that, with respect to the occurrence of isotope dilution effects, a distinction should be made between ^{65}Zn administration in an aqueous solution to fastened animals and ^{65}Zn administration in food. We found that in mice fed a diet just adequate in Zn (22 µg Zn/g) the absorption of ^{65}Zn from water, administered by intragastric intubation after 20 hours of fasting, was constant (about 60%) for doses from 0.3 up to 30 µg Zn at least. At higher doses, which were not included in the experiments, ^{65}Zn absorption would probably decrease, as can be derived from results of Jackson et al. [4]. Absorption of ^{65}Zn from food, in contrast, was found to decrease gradually from 53% to 19%, when the dose increased from 1.5 to 100 µg Zn.

On the ground of these results we concluded that, when ^{65}Zn is administered in water, isotope dilution by endogenous luminal zinc is not likely to have an effect on the absorption of ^{65}Zn, unless the endogenous luminal Zn pool would contribute more than 30 µg Zn to the ^{65}Zn dose, which is half the daily requirement. However, when ^{65}Zn is administered in food, isotope dilution by endogenous luminal zinc could cause a reduction of the ^{65}Zn absorption, because ^{65}Zn absorption from food appears to be dose dependent. The

reason for this food effect on dose dependence is not well understood.

Another aspect of intraluminal isotope dilution should also be considered. Weigand and Kirchgessner [5] have shown that in rats fed a diet just adequate (15-20 µg/g) or deficient (<15 µg/g) in Zn the endogenous excretion of this element occurs at a minimum level, required for maintenance, in rats fed a Zn replete diet (>>20 µg/g) the endogenous excretion rapidly increases with the Zn concentration in the diet due to homeostatic mechanisms. Flanagan et al. [1] found 0.4 µg Zn in the lumen of mice fed the zinc-deficient diet (1.0 µg Zn/g) and 8.9 µg Zn in the lumen of mice fed the zinc-replete diet (48.5 µg Zn/g). Therefore, the finding in rats probably also applies to mice. This would mean that in mice fed a diet just adequate in Zn the endogenous luminal Zn pool is as low as in zinc-deficient mice, viz., 0.4 µg Zn, and that as a consequence isotope dilution in such mice probably is negligible.

This hypothesis is supported by our observation of a dose dependence of ^{65}Zn absorption from food in mice fed a diet just adequate in Zn, similar to that found by Flanagan et al. in zinc-deficient mice. Therefore, we like to suggest that in mice a significant isotope dilution may occur only when a zinc-replete diet is fed and that the effect of such isotope dilution on ^{65}Zn absorption depends on the degree of dilution and on the ^{65}Zn carrier (water or food).

Literature cited

1. Flanagan, P.R., Haist, J. & Valberg, L.S. (1983) Zinc absorption, intraluminal zinc and intestinal metallothionein levels in zinc-deficient and zinc-replete rodents. J. Nutr. 113, 962-972.
2. Evans, G.W., Johnson, E.C. & Johnson, P.E. (1979) Zinc absorption in the rat determined by isotope dilution. J. Nutr. 109, 1258-1264.
3. Van Barneveld, A.A. & Van den Hamer, C.J.A. (1984) Influence of trace element concentration on absorption and retention of trace element radiotracers in mice, a study with ^{75}Se, ^{64}Cu, ^{65}Zn and ^{203}Pb. To be published.
4. Jackson, M.J., Jones, D.A. & Edwards, R.H.T. (1981) Zinc absorption in the rat. Br. J. Nutr. 46, 15-27.
5. Weigand, E. & Kirchgessner, M. (1978) Homeostatic adaptation of Zn absorption and endogenous Zn excretion over a wide range of dietary Zn supply. In: Trace element metabolism in man and animals-3 (TEMA-3), pp. 106-109 (Ed. M. Kirchgessner), ATW Freising-Weihenstephan, BRD.

To be sent to the Journal of Nutrition as a Letter to the Editor.

APPENDIX III

MAIN EFFECTS OF CA AND MG ON METABOLISM OF MN, PB AND CD IN MICE

SUMMARY The epidemiologically observed relationship between drinking water hardness and mortality from cardiovascular disease has been attributed, among others, to an increased uptake of toxic elements from soft drinking water. There are two possible reasons, why this uptake could be increased in soft water regions: (1) increased dissolution of toxic metals from the distribution system; (2) increased absorption of toxic metals in the gastrointestinal tract. This report focuses on the possibility that the drinking water hardness factors Ca and Mg protect against the uptake of the metals Mn, Pb and Cd from the gut. In mice both Ca and Mg were found to protect against the absorption of Mn from an aquous solution. The absorption of Pb was inhibited only by Ca. The absorption of Mn, Pb and Cd was stimulated by Ca-deprivation during two weeks. In soft water regions an increased absorption of Mn and Pb can be expected, especially when the diet is marginal in Ca.

Introduction.

During a 4 years research project the influence of Ca and Mg on the absorption of a range of trace elements, including Cu, Zn, Co, Se, Mn, Pb and Cd, has been investigated in mice. The ultimate goal of this project was to elucidate some aspects of the epidemiologically found relation between drinking water hardness and cardiovascular disease, the so-called "water story" [1]. Our attention has been focused on the possibility of an altered trace element absorption from the gut in soft water regions due to the low concentration of Ca and Mg in the drinking water. Earlier the results obtained with the elements Cu, Zn, Co and Se and preliminary results on Cd were reported [2]. In this paper the major results obtained with Mn, Pb and Cd will be presented.

Methods.

The following two experiments were carried out:

Experiment 1: 3x3 groups of 10 female Swiss Random mice, 4 weeks old, were fed during 2 weeks a purified diet (IRI-CB), especially composed for trace element research [3]. After 20 hours of fasting the mice received 0.3 ml of a solution containing 1 µCi of ^{54}Mn (1 µg Mn/ml) or ^{203}Pb (1 µg Pb/ml) or ^{115m}Cd (15 µg Cd/ml), as the chloride, by intragastric intubation. Three types of solution were used, viz., demineralized water as such, or supplemented with $CaCl_2$ (25 mM), or $MgCl_2$ (25 mM). The retention of the isotopes was measured by whole-body counting during 9-12 days. During this period the mice were fed the IRI-CB diet, starting 6 hours after the dose.

Experiment 2: 4x3 groups of 11 female Swiss Random mice, 4 weeks old, were

fed during 2 weeks a Ca,Mg-poor diet (IRI-CB/P diet = IRI-CB diet without Ca- and Mg-salts) as such, or supplemented with $CaCO_3$ (0.4% Ca), or $MgSO_4\,7H_2O$ (0.05% Mg), or both (0.4% Ca + 0.05% Mg). The composition of the last diet was exactly the same as that of the standard IRI-CB diet. After this period on the test diets the mice were fasted 20 hours. Then they received 0.3 ml demineralized water containing 1 µCi of ^{54}Mn (1 µg Mn/ml) or ^{203}Pb (1 µg Pb/ml) or ^{115m}Cd (15 µg Cd/ml), as the chloride, by intragastric intubation. The retention of the isotopes was measured during 9-12 days. During this period the mice were fed the IRI-CB/P diet without supplements, starting 6 hours after the dose.

The apparent absorption (A_a) of the isotopes was measured according to the method of Heth and Hoekstra [4] by extrapolation of the linear part of the semilogarithmic retention curve to the ordinate and determination of the point of intersection.

Results.

Table 1 shows the influence of Ca and Mg, added to the dose, on the absorption of ^{54}Mn, ^{203}Pb and ^{115m}Cd from an intubated aqueous solution in mice. The absorption of Mn was reduced by both Ca and Mg (64% and 47%, resp.). The absorption of Pb was only reduced by Ca (62%). This reduction had a low significance ($P<0.10$) due to the high variation of the absorption of

Table 1 The direct influence of Ca and Mg (25 mM) on the apparent absorption of ^{54}Mn, ^{203}Pb and ^{115m}Cd from an intubated solution.

	^{54}Mn (%)	^{203}Pb (%)	^{115m}Cd (%)
Control	5.4±1.4	11.0±10.6	4.3±1.7
Ca (25 mM)	1.9±0.7 **	4.2± 2.5 (*)	3.4±1.0
Mg (25 mM)	2.8±1.0 **	11.2± 6.8	4.2±1.0

Table 2 The influence of chronic Ca- and Mg-deprivation on the apparent absorption of ^{54}Mn, ^{203}Pb and ^{115m}Cd from an intubated solution.

	^{54}Mn (%)	^{203}Pb (%)	^{115m}Cd (%)
Ca,Mg-depr.	2.7±1.5 **	36.7±13.0 **	3.9±0.8
Mg-depriv.	1.2±0.4	27.9±15.5	3.4±0.8
Ca-depriv.	2.5±0.6 **	40.2±15.8 **	4.1±0.7 *
Control	1.3±0.3	18.4±11.6	3.6±0.4

**, * and (*): significantly different from control by Student's t-test, $P< 0.01$, 0.05, and 0.10, resp.

^{203}Pb in the control group. The absorption of Cd was also reduced by Ca, but the effect was smaller (22% and not significant.

Table 2 shows the influence of the Ca- and Mg-status of mice on the absorption of ^{54}Mn, ^{203}Pb and ^{115m}Cd from an aqueous solution. The Ca-deprived mice, viz., the mice fed the Ca,Mg-poor or the Ca-poor diet, showed a 100% increase in the absorption of Mn and Pb, and a 10% increase in the absorption of Cd which was not significant in the Ca,Mg-deprived mice. Mg-deprivation as such had no significant effect on absorption.

Discussion.

Three major factors have been mentioned as possible causes of the relationship between drinking water hardness and cardiovascular disease: (1) suboptimal Ca- or Mg-intake in soft water regions; (2) increased solubilization of toxic metals from the distribution system by soft water; (3) protection against the absorption of toxic metals from the drinking water by the water hardness factors Ca and Mg. Our results show that both Ca and Mg may protect against the uptake of Mn, and that Ca may protect against the uptake of Pb. The decrease of the Cd-absorption by Ca was small and not significant.

The mechanism of Mn-absorption is unknown. The direct interaction between Ca and Mn and between Mg and Mn suggests a competition for a common step in the transport mechanism in the mucosal cell, probably that which is involved in the competition between Ca and Mg for absorption [5].

The interaction between Ca and Pb may occur on another level, because Mg is not participating in the interaction and because Pb is a much larger ion than Ca and Mn. Barton et al [6], however, have suggested that Pb and Ca may compete for mucosal receptors necessary for their passage across the mucosal cell. They found that calcium binding protein (CaBP) and a high molecular weight protein (HMWP) could bind both Ca and Pb and thus may play a role in the interaction between Ca and Pb. In in vitro experiments with everted gut sacs Hilburn et al [7] found an increased Pb-absorption in total absence of Ca. They suggested that the tight junctions and desmosomes between the mucosal cells need Ca to prevent extracellular transport of Pb from the lumen to the circulation. However, in vivo a total absence of Ca in the lumen is not likely because of endogenous excretion of Ca in the digestive juice. More research is needed to clarify the mechanism of the Ca-Pb interaction.

Another interesting aspect is the influence of chronic Ca-deprivation on the absorption of trace metals. Ca-deprivation (combined with or without Mg-deprivation) increased the absorption of Mn and Pb and slightly that of Cd. In Ca-deprived mice the endogenous excretion of Ca may be strongly reduced. Therefore the luminal content of Ca at the moment of intubation of the isotope solution may be lower in the Ca-deprived mice than in the control mice. According to the results of experiment 1, this effect may explain the increase of the absorption of both ^{54}Mn and ^{203}Pb and, if the small decrease

of ^{115m}Cd absorption by Ca was a true effect, also that of ^{115m}Cd.

There may be a additional cause for the observed effects in Ca-deprived mice It has been shown [8] that Ca-deprivation increases Ca-absorption by a mechanism, in which calcitriol is involved. Calcitriol, a vitamin D derivative, stimulates the synthesis of CaBP and is also responsible for a facilitated diffusion of Ca through the mucosa membrane [9] The increased permeability of the cell membrane may be non-specific and other elements like Mn, Cd and Pb might take advantage of this to enter the cell. However, on basis of the observation that the absorption of Cu, Zn and Co in mice is not increased by Ca-deprivation [2] this hypothesis seems less likely. In the cell an increased binding of metal to CaBP might perhaps play a role, especially for Pb and Cd.

As concerned the "water story" it is concluded, that water hardness (Ca + Mg) protects against the uptake of Mn and Pb. In soft water regions this protection is absent. A low dietary Ca-intake would further stimulate Mn- and Pb-uptake. An increased uptake of Mn and Pb may perhaps not be a risk factor for cardiovascular disease However, it might become one in combination with, for example, a low dietary Mg-intake. A substitution of Mn for Mg in the mitochondria of the heart muscle may disturb energy processes and cause arrythmia and "sudden death" [10]. A similar effect may occur by combination of Pb-absorption with a low Mg-intake or with some other factor related to soft water intake.

These results indicate that water hardness may protect against the absorption of (potentially toxic) Mn and Pb. It is suggested that the toxicity of these metals may be potentiated by a low Mg-intake or another soft-water-factor.

References.

1. Punsar, S (1973) Work-environ.-health 10, 107.
2. Van Barneveld, A.A., Van den Hamer, C.J.A. and Houtman, J.P.W. (1982) Tr. Subst. Environ. Hlth 16, 196.
3. Van Barneveld, A.A. and Morse, H. (1981) 1st Int. Sci. Meet FELASA, Düsseldorf, BRD, June 1981 (IRI-rapport 133-81-02, Delft, The Netherlands).
4. Heth, D.A. and Hoekstra, W.G. (1965) J. Nutr. 85, 367.
5. Hendrix, J.Z., Alcock, N.W. and Archibald, R.M. (1963) Clin.Chem. 9, 734.
6. Barton, J.C., Conrad, M.E., Harrison, L. and Nuby, S. (1978) J Lab. Clin. Med. 91, 366.
7. Hilburn, M.E., Coleman, I.P.L. and Blair, J.A. (1980) Environ. Res. 23, 301.
8. Allen, L.H. (1982) Amer. J. Clin. Nutr. 35, 783.
9. Anonymous (1978) Nutr. Rev. 36, 90.
10. Marienfeld, C.J. and Collins, M. (1981) Tr. Subst. Environ. Hlth 15, 3.

APPENDIX IV

The experiments described in Section 5.2-5.4 were all relatively short-term experiments. As a pilot study a long-term experiment was performed, which did not yield results suitable for publication in the open literature. Description of the experiment, however, could serve as a starting point for further research.

EFFECT OF LONG-TERM ADMINISTRATION OF CA, MG OR NA IN DRINKING WATER ON TRACE ELEMENT ACCUMULATION FROM FOOD, ON SERUM MINERAL AND LIPID COMPOSITION AND ON BEHAVIOUR IN MICE.

SUMMARY To investigate the influence of long-term administration of minerals in drinking water on some aspects of mineral, lipid, and trace element metabolism, male and female Swiss mice were placed on a regimen of stock diet and of drinking water containing Ca, Mg or Na (5, 5, and 10 µmole/ml, resp.) during 8 and 12 months, respectively. These mice are indicated as Ca-group, Mg-group, and control group, respectively. After sacrificing, no effects of the water composition were observed on the mineral content of the serum of the mice, except for a decreased Mg concentration in the serum of the male mice of the Mg-group compared to that of the control group. In the male mice of the Ca- and Mg-group the total serum lipids and the serum cholesterol tended to be reduced. In the female mice of the corresponding groups the total serum cholesterol and the HDL-cholesterol tended to be reduced. In these mice practically all cholesterol was found in the HDL-fraction, whereas in the male mice the HDL-fraction contained only 70% of the cholesterol. The trace element composition of some selected organs of the female mice showed decreased levels of Cd in the liver and Fe in the kidneys in the Ca-group. In the Mg-group decreased levels of Cu in the liver and Se in the kidneys and an increased level of Co in the heart were found. The possible health effects of these changes in trace element contents of these organs are not clear, except for the lower Cd content of the liver in the Ca-group. This could indicate a protective effect of drinking water hardness against chronic low levels of Cd intake. Surprisingly, in the male mice an adverse effect of Ca and Mg in the drinking water on behaviour was found. The Ca-group and even more the Mg-group was aggressive compared to the control group. The female mice were not aggressive at all. It is pointed out that the observed effects were caused by a relatively small contribution of minerals from the drinking water compared to the total dietary intake. Drinking water might be more important as a source of dietary Ca and Mg than would be expected on account of its relative contribution to the total intake.

Table 1 Trace element composition of SRMA stock diet.

Tr.el.	µg/g	Tr.el.	ng/g
Fe	196	Ni	1.1
Zn	92	As	0.8
Mn	70	Se	0.5
Cu	25	I	0.35
Cr	1.6	Co	0.3
Mo	1.5	Cd	0.17

Introduction

Many papers have reported a negative correlation between drinking water hardness and mortality from cardiovascular disease [1]. Although there has been intensive research on the possible cause, no single or simple "water factor" has been identified [2]. An influence of Ca and Mg in the drinking water on the absorption of trace elements and on lipid metabolism has often been mentioned as of possible importance.

In the present report some effects will be described of chronic administration of Ca and Mg, compared to Na, in the drinking water of mice on the accumulation of trace elements from the food in liver, kidneys and heart and on serum mineral and lipid composition. Because of limited analytical capacity it was necessary to minimize the number of analyses by pooling samples. As a result the data do not allow statistical evaluation. The experiment, however, was meant to detect tendencies that could be related to short-term experiments showing an influence of Ca or Mg on trace element absorption [3], and that could be used as indications for further research. During the experiment a change in behaviour of male mice was observed, which will be described as well.

Methods

3 Groups of 20 male and 3 groups of 30 female Swiss Random mice were obtained from the Central Institute for the Breeding of Laboratory Animals - TNO, Austerlitz, The Netherlands, at the age of 4 weeks. They were housed in macrolon cages with stainless steel lids and glass drinking bottles with glass nipples. For a long-term drinking water experiment they were placed on a regimen of stock diet (SRMA from Hope Farms, Woerden, The Netherlands; the trace element composition is given in Table 1) and drinking water containing 5 µmole $CaCl_2$/ml, 5 µmole $MgCl_2$/ml, or 10 µmole NaCl/ml. The three groups will be indicated as the Ca-group, the Mg-group, and the control group. The

stock diet contained 96 µmole Na/g, 230 µmole Ca/g, 95 µmole Mg/g, 190 µmole K/g and 230 µmole P/g.

After 8 months the male mice and after 12 months the female mice were sacrificed. Blood was sampled through the plexus orbitalis and pooled per group of 5 male mice and per group of 10 female mice, thus producing 4 and 3 samples per Ca-, Mg- or Na-group of male and female mice, resp. After clotting and centrifugation the sera were collected and used for analysis of minerals (Ca, Mg, Na and K) and lipids (total cholesterol, HDL-cholesterol, triglycerides and total lipids).

Liver, kidneys, heart and bone of the female mice were dissected with special metal-contamination free tools (a wolfram-carbide knife and titanium scissors and tweezers) in a dust-free room. The soft tissues were homogenized using an alabaster morter, pooled per group of 10 mice, freeze-dried and analyzed together with the pooled femurs and sera of the female mice by neutron activation according to the method of Tjioe et al. [4] for the trace elements Cu, Zn, Se, Co, Fe, Mo and Cd.

Results

The serum Ca, Mg, Na and K levels are shown in Table 2. Na and Mg tended to be lower, Ca and K higher in the serum of the female mice compared to the male mice. Serum Mg tended to be lower in the male mice of the Mg-group.

The results of the analyses of lipids in the sera are shown in Table 3. Differences both between male and female mice and between the Ca- and Mg-groups and the control group were observed. In the male mice total serum lipids and serum cholesterol tended to be reduced in the Ca- and Mg-groups, whereas in the corresponding groups of the female mice serum cholesterol and HDL-cholesterol tended to be reduced. In the male mice the HDL-fraction contained about 70% of total cholesterol; in the female mice this fraction contained practically all cholesterol.

Table 4 shows the content of 7 trace metals in liver, kidneys and heart of the female mice. Liver Cd and kidney Fe tended to be lower in the Ca-group. Liver Cu and kidney Se tended to be lower and heart Co higher in the Mg-group. The data on serum and femurs were very inconsistent and poorly reproducible or close to the detection limit. They are not reported because of their low reliability. Only the data on Cu and Zn in serum are presented (Table 5); they show no influence of the water composition.

In the course of the experiment an increasing tendency to fight was observed in the male mice. The degree of aggressiveness differed between the groups. It was low in the control group, higher in the Ca-group and highest in the Mg-group. After 8 months continuation of the experiment with these mice was no longer acceptable, so they were sacrificed. In the aggressive Ca-group and the still more aggressive Mg-group the mice were found to have

Table 2 Serum minerals in male and female mice after long-term administration of Na, Ca, or Mg in drinking water.

Water	Na (mmol/l)	Ca (mmol/l)	Mg (mmol/l)	K (mmol/l)
a. Males [1]				
10 mM Na^+	157 ± 1	2.24 ± 0.09	1.41 ± 0.09	9.4 ± 1.4
5 mM Ca^{++}	156 ± 6	2.22 ± 0.04	1.36 ± 0.03	9.6 ± 0.1
5 mM Mg^{++}	164 ± 8	2.37 ± 0.13	1.18 ± 0.07	9.4 ± 0.9
b. Females [2]				
10 mM Na^+	151 ± 1	2.43 ± 0.04	1.28 ± 0.07	12.0 ± 1.0
5 mM Ca^{++}	153 ± 1	2.43 ± 0.06	1.28 ± 0.10	11.5 ± 0.4
5 mM Mg^{++}	150 ± 2	2.41 ± 0.05	1.20 ± 0.11	11.5 ± 0.4

[1] Blood was pooled per 5 mice; data are given as means ± sd of the 4 pooled sera collections obtained.

[2] Blood was pooled per 10 mice; data are given as means ± sd of the 3 pooled sera collections obtained.

Table 3 Serum lipids in male and female mice after long-term administration of drinking water containing Na, Ca or Mg. [3]

Water	Chol.-total (mmol/l)	Chol.-HDL (mmol/l)	% [4]	Triglyc. (mmol/l)	Total lipids (g/l)
a. Males [1]					
10 mM Na^+	2.6 ± 0.1	1.8 ± 0.1	71	0.7 ± 0.1	5.5 ± 0.1
5 mM Ca^{++}	2.3 ± 0.2	1.7 ± 0.1	74	0.7 ± 0.2	5.0 ± 0.4
5 mM Mg^{++}	2.2 ± 0.4	1.6 ± 0.2	71	0.5 ± 0.1	4.5 ± 0.6
b. Females [2]					
10 mM Na^+	2.0 ± 0.4	2.0 ± 0.4	99	1.2 ± 0.4	- [5]
5 mM Ca^{++}	1.5 ± 0.2	1.5 ± 0.1	99	0.9 ± 0.1	- [5]
5 mM Mg^{++}	1.7 ± 0.2	1.5 ± 0.1	88	1.3 ± 0.5	- [5]

[1, 2] As in Table 2.

[3] The analyses of sera from male and from female mice were performed at two different laboratories. Comparison within the male groups and within the female groups is allowed.

[4] HDL-cholesterol as fraction of total cholesterol.

[5] Data on serum of females are not available.

a 2- to 3-fold enlarged spleen compared to the Na-group; mean spleen weights were 0.12 g, 0.17 g, and 0.07 g, resp. The groups of female mice, none of which were aggressive, were sacrificed at the age of 12 months. In these mice no spleen enlargement was found (0.07 g for all groups).

Discussion

The three types of drinking water administered to the mice contained 5 µmole Ca/ml, 5 µmole Mg/ml, or 10 µmole Na/ml, respectively. The value for Ca was slightly higher than is found in very hard drinking waters in some areas in our country [5]. The concentrations of Na and Mg were chosen to provide the same amount of chloride as in case of Ca addition. This was also the reason for using Na-containing drinking water as control.

The stock diet contained rather high levels of the minerals in question, viz., 9300 µg Ca/g, 2300 µg Mg/g and 2200 µg Na/g. Assuming that the food consumption in g was approximately equal to the drinking water consumption in ml, the contribution of the drinking water to the total mineral intake was relatively small (2%, 5%, and 10%, resp.).

The differences observed in serum minerals and lipids of male and female mice (Table 2 and Table 3) could be caused by sex difference and by difference in age, because at the moment of sacrifice the female mice were 4 months older than the males. The differences in serum lipids could also have an analytical cause, because the sera of the male and female mice were analyzed in two different laboratories.

Serum minerals in general were not influenced by the drinking water composition. This was not surprising because the homeostatic mechanisms which regulate these are very efficient. The lower serum Mg of the male mice of the Mg-group is not understood.

Serum lipids showed both in male and in female mice a tendency to be reduced in the Ca- and Mg-groups. This could be the result of a decreased absorption of fatty acids due to formation of Ca or Mg salts, as has been found in rats [6]. This seems in accordance with work of Dougherty and Iacono [7] who found elevated cholesterol and phospholipid levels in plasma of rabbits fed a Ca-deficient diet. Similar results were reported by Rayssiguier et al. [8] who observed increased plasma triglyceride levels and a shift of cholesterol from the HDL to the LDL fraction in Mg-deficient rats. In contrast, in the present study a reduction of HDL-cholesterol was observed by Ca and Mg supplementation in female mice. This reduction, however, was not accompanied by an increase in LDL-cholesterol. Also in man, serum cholesterol and triglycerides have been found to be reduced by increased dietary Ca [9]. In this connection it is interesting to realize that a high LDL-cholesterol in the plasma is considered a risk factor in cardiovascular disease [10].

Table 4 Trace element concentrations (µg/g or ng/g wet weight) in liver, kidneys and heart of female mice after long-term administration of drinking water containing Na, Ca or Mg. [1]

	Cu	Zn	Se	Co	Fe	Mo	Cd
Liver	µg/g	µg/g	µg/g	ng/g	µg/g	µg/g	ng/g
10 mM Na^+	5.4±1.2	34±3	1 8±0.3	58± 5	300±60	0.9 ±0 2	73±12
5 MM Ca^{++}	4.4±0.7	33±5	1.7±0.2	60±10	300±50	1.0 ±0.3	44± 9
5 mM Mg^{++}	3.0±0.5	33±2	1.5±0.2	63±10	290±30	0.8 ±0.1	68±21
Kidneys							
10 mM Na^+	4.2±2.4	22±1	2.0±0.1	190±17	150±20	0.4 ±0.1	270±30
5 mM Ca^{++}	3.1±1.4	20±1	1.8±0.4	181±31	120±10	0.4 ±0.1	280±50
5 mM Mg^{++}	5.1±1.6	21±1	1.7±0.1	199±24	170±30	0.3 ±0.1	260±40
Heart							
10 mM Na^+	6.9±2.1	18±1	0.4±0.1	32± 2	139± 4	0.05±0.01	-- [2]
5 mM Ca^{++}	5.8±1.0	18±4	0.4±0.1	35±11	143±15	0.05±0.01	-- [2]
5 mM Mg^{++}	6.4±1.3	19±2	0.4±0.1	40± 5	145±24	0.05±0.02	-- [2]

[1] Organs were pooled per 10 mice; data are given as means±sd of the three values obtained per organ.

[2] Data below detection limit.

Table 5 Cu and Zn concentrations (µmole/l) in serum of female mice after long-term administration of drinking water containing Na, Ca or Mg.

Serum	Cu (µmole/l)	Zn (µmole/l)
10 mM Na^+	15±2	16±1
5 mM Ca^{++}	14±4	18±2
5 mM Mg^{++}	12±2	18±5

The influence of the drinking water composition on the trace element content of the organs (Table 4) was most pronounced in the Mg-group. Compared to the Na- and Ca-group, Cu showed a shift from the liver to the kidneys. Se was low in both liver and kidneys and Co was high in the heart. In the Ca-group the low content of Cd in the liver was remarkable. In short-term experiments using radiotracers of trace elements it was found that Ca and Mg in the drinking water could reduce the absorption of Cd from food [3]. The low Cd content in the liver of the Ca-group could therefore be the result of a reduction of the Cd absorption. Because absorbed Cd is accumulated in the organs (liver, kidneys), probably bound to metallothionein, and thus Cd should have a very long half-life (about 16 years) [11], a small reduction of the absorption could on the long run result in a large difference in the body burden of Cd.

The possible health effects of the observed changes in the trace element content of the organs are not clear. Trace elements are mentioned in relation to heart disease and cancer [12]. The mechanisms underlying such relationships are insufficiently known to conclude that one of the observed effects could be indicative for a detrimental or protective effect of drinking water hardness. An exception should be made for Cd because of its high toxicity and its tendency to accumulate in the body. The binding to metallothionein in the organs, however, seems to have a detoxifying effect. On the other hand, chronic low level administration of Cd has been reported to produce hypertension [13,14] - although this subject is controversial [15,16] - , changes in cardiac physiology [13] and atherosclerosis in rats [14]. A protective role of drinking water hardness, e.g., through reduction of Cd absorption from food [3], should be considered. Revis et al. [14], however, reported an antagonistic effect of Mg as part of water hardness on the protective effect of Ca in hard drinking water. This subject requires further investigation.

There was a clear difference in the aggressive behaviour of the male mice in the three drinking water treatment groups. This finding was not accidental; in two similar experiments using male mice the same difference in behaviour was observed. The higher aggressiveness of the mice given Ca and particularly of those given Mg in the drinking water was reflected in the severity of bite wounds in their tail, back and ears. The spleen enlargement which was found in the mice belonging to the more aggressive groups and which seemed to be related to the observed degree of aggressiveness, could have resulted from infectious processes caused by the bite injuries.

It is not understood how Na, Ca or Mg can relate to aggressive behaviour. Aggressiveness in the Ca- and Mg-treated mice was relative to the more tolerant Na-treated mice, so it can not be excluded that the Mg-treated mice behaved normally (aggressiveness of male mice is not exceptional) and

that Na, and to a lesser extent Ca, has an aggression suppressing effect in the male mice The observations, however, offer no possibility for identification of an aggression stimulating or reducing factor.

The Ca and Mg concentration of the drinking water was high, but of the same order as that found in some areas in this country. From a nutritional point of view it is important that this relatively small contribution (2% and 5% for Ca and Mg, resp) of minerals in the drinking water to the dietary intake can cause the observed effects. The water contribution to the total amount of minerals absorbed from the diet could therefore be relatively high due to a high availability of water minerals compared to food minerals. In rats it was found [17], that the fractional absorption of Ca from an aqueous solution was more than two times higher than the fractional absorption of Ca from food. We could confirm these results in mice (unpublished results). Because Ca and Mg are biochemically strongly related, it is likely that also Mg is much better absorbed from drinking water than from food. Drinking water might be more important as a source of dietary calcium and magnesium than would be expected on account of its relative contribution to the total intake.

Acknowledgements

Prof.ir. J.P.W. Houtman and Dr. J.H.M. Van Tongeren are acknowledged for critical review of the manuscript. Dr. P.S. Tjioe and Mr. J. Schuite are acknowledged for neutron activation analyses of mouse tissues and Dr Postma and Dr. T. Hoogenraad for lipid and mineral analyses of the sera. Ms. H.K. Terlouw is thanked for technical assistance.

References

1. Punsar, S. (1973) Cardiovascular mortality and quality of drinking water. An evaluation of the literature from an epidemiological point of view. Work-environm.-hlth 10, 107-125.
2. Proc. Symp. "Drinking water and cardiovascular disease" (1980) J. Environm. Pathol Toxicol. 4, 5-326.
3. Van Barneveld, A.A., Van den Hamer, C.J.A. & Houtman, J.P.W (1982) Drinking water hardness, trace elements and cardiovascular diseases: Influence of Ca and Mg on metabolism of Cd, Co, Cu, Zn and Se in mice. Tr. Subst. Environ. Hlth 16, 196-204.
4. Tjioe, P.S., De Goeij, J.J.M. & Houtman, J.P.W. (1977) Extended automated separation techniques in destructive neutron activation analysis; application to various biological materials, including human tissues and blood. J. Radioanal. Chem. 37, 511-522.
5. Haring, B.J.A. & Zoeteman, B.C.J. (1980) Corrosiveness of drinking water

and cardiovascular disease mortality. Bull. Environm. Contam. Toxicol. 25, 658-662.

6. Mattson, F.H., Nolen, G.A. & Webb, M.R. (1979) The absorbability by rats of various triglycerides of stearic and oleic acid and the effect of dietary calcium and magnesium. J. Nutr. 109, 1682-1687.
7. Dougherty, R.M. & Iacono, J.M. (1979) Effects of dietary calcium on blood and tissue lipids, tissue phospholipids, calcium and magnesium levels in rabbits fed diets containing beef tallow. J. Nutr. 109, 1934-1945.
8. Rayssiguier, Y., Gueux, E. & Weiser, D. (1981) Effect of magnesium deficiency on lipid metabolism in rats fed a high carbohydrate diet. J. Nutr. 111, 1876-1883.
9. Yacowitz, H. (1976) Increased dietary calcium reduces serum cholesterol, triglycerides in humans. Feedstuffs 48, 27.
10. Havel, R.J. (1979) High-density lipoproteins, cholesterol transport and coronary heart disease. Circulation 60, 1-3.
11. Ellis, K.J., Vartsky, D., Zanzi, I., Cohn, S.H. & Yasumara, S. (1979) Cadmium, In vivo measurement in smokers and nonsmokers. Science 205, 323-324.
12. Anonymous (1976) Heart disease, cancer linked to trace metals. Chem. Engin. News 3, 24-27.
13. Kopp, S.J., Perry, Jr., H.M., Perry, E.F. & Erlanger, M. (1983) Cardiac physiologic and tissue metabolic changes following chronic low-level cadmium and cadmium plus lead ingestion in the rat. Toxicol. Appl. Pharmacol. 69, 149-160.
14. Revis, N.W, Zinsmeister, A.R. & Bull, R. (1981) Atherosclerosis and hypertension induction by lead and cadmium ions: An effect prevented by calcium ion. Proc. Nat. Acad. Sci. USA 78, 6494-6498
15. Porter, M.C., Miya, T.S. & Bousquet, W.F. (1974) Cadmium: Inability to induce hypertension in the rat. Toxicol. Appl. Pharmacol. 27, 692-695.
16. Doyle, J.J., Bernhoft, R.A. & Sandstead, H.H (1976) The effects of a low level of dietary cadmium in rats. Proc. N.D. Acad. Sci. 28, Pt. 2, 67-78.
17. Marcus, C.S. & Langemann, F.W. (1962) Absorption of Ca^{45} and Sr^{85} from solid and liquid food at various levels of the alimentary tract of the rat. J. Nutr. 77, 155-160.

STELLINGEN

1. De betekenis van essentiële spoorelementen voor het functioneren van het lichaam evenaart die van vitaminen.

2. In stofwisselingsexperimenten met spoorelementen waarbij gebruik gemaakt wordt van isotopen, dient rekening gehouden te worden met het optreden van effecten als gevolg van isotoopverdunning.

   dit proefschrift (app. II)

3. Speciale diëten voor proefdieren dienen met de grootste zorgvuldigheid te worden samengesteld en bereid.

   dit proefschrift (4.2)

4. In het algemeen zal een hoog gehalte aan fytaat en vezelstoffen in de Nederlandse voeding geen aanleiding geven tot een tekort aan spoorelementen.

   dit proefschrift (app. I)

5. Uit een verlaagde retentie van oraal toegediend ^{54}Mn in ratten, die voordien gevoed zijn met een dieet met verhoogd Mn gehalte, mag niet zonder meer geconcludeerd worden dat de absorptie van Mn gereguleerd wordt als onderdeel van de Mn homeostase.

   Abrams, E., Lassiter, J.W., Miller, W.J., Neathery, M.W., Gentry, R.P. and Scarth, R.D. (1976) J.Animal Sci. 42, 630-636.

6. De juistheid van het recentelijk opnieuw gegeven advies een Ca-arme voeding aan te vullen met melk om opname van Pb uit het voedsel tegen te gaan, dient te worden betwijfeld.

   Blake, K.C.H. and Mann, M. (1983) Environ. Res. 30, 188-194.

7. De resultaten van reacties van CH_xCl_{4-x} (x=1-3) en H_2 over FT-katalysatoren leveren een bijdrage tot een beter inzicht in het mechanisme van de Fischer-Tropsch synthese.

   Van Barneveld, W.A.A. (1983) Proefschrift, Leiden 19 mei 1983.

8. In de fundamentele wetenschap zijn foute interpretaties, mits als hypothese geformuleerd, even waardevol als juiste interpretaties.

9. Bij de overdracht van medische analysegegevens van het laboratorium naar de medicus dienen beide partijen zorg te dragen voor een goed inzicht in de numerieke waarde en de betekenis van de betreffende gegevens.

10. De financiële begroting van uit te voeren wetenschappelijk onderzoek wordt mede bepaald door de ter beschikking staande financiële middelen

11. Chelatie-therapie heeft vooral voordelen voor de therapeut.

12. Het uitlaten van honden op speelterreinen voor kinderen getuigt van onnadenkendheid.

13 De voortgaande bewapening der machtsblokken in de wereld is ook niet met goede argumenten te rechtvaardigen.

14. Meditatie is een verantwoorde en uiterst efficiënte vorm van zelf-ontwikkeling, mits de gevolgde methode de beoefenaar met beide benen op de grond houdt.

15. Het begrip liefde wordt in het algemeen uiterst beperkt geïnterpreteerd.

A.A. van Barneveld, januari 1984